



# Role of the Capsid Helix 4-5 Loop in Equine Infectious Anemia Virus Infection

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## **Role of the Capsid Helix 4-5 Loop in Equine Infectious Anemia Virus Infection**

### **Abstract**

The lentiviral capsid core, which encapsulates the viral RNA genome, is delivered into the target cell cytoplasm during the viral entry process. In the cytoplasm, the conical core undergoes morphological changes, which are termed uncoating. Proper uncoating has been shown to be critical for the infectivity of the lentivirus HIV-1. In addition, the HIV-1 capsid protein is critical for the process of nuclear import of the preintegration complex (PIC). The lentivirus equine infectious anemia virus (EIAV) shares many similarities to HIV-1, including similarities in the capsid protein. In particular, both HIV-1 and EIAV capsid contain a proline-rich loop region in the amino terminal domain of capsid between helices 4 and 5. The host cellular factor cyclophilin A binds this loop in HIV-1 and is critical for proper uncoating. We hypothesized that this helix 4-5 loop was also critical for EIAV infectivity at some early step in the viral infection cycle. We created a panel of amino acid substitution mutations in this loop region. Some of the mutations resulted in severely deleterious effects on EIAV infectivity. Some mutations caused a slight increase in infectivity. The deleterious mutations did not affect uncoating or reverse transcription but appeared to block nuclear import of the PIC. Those mutations in which infectivity was slightly increased did not exhibit significantly different phenotypes from wild-type EIAV at any of the stages examined. The results of this study lend further support to the role of capsid as a determinant of nuclear import and suggest that viral and cellular factors critical to HIV-1 import may also be applicable to

EIAV. Future research should focus on identifying the causes of the defects in nuclear import observed for some mutants, as well as attempt to identify the reason for the infectivity increase in others. In addition, inclusion of EIAV in future studies of nuclear import involving HIV-1 can broaden the scope of the data to lentiviruses in general rather than HIV-1 in particular.

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# CHAPTER 1

## **INTRODUCTION**



The *Retroviridae* family consists of a large group of viruses that undergo an unusual step during their infection cycle, from which they derive their name. Virions contain an RNA genome, but during infection this RNA is copied into DNA: a process termed reverse transcription. This flow of genetic material is in the opposite direction of that termed the central dogma (DNA → RNA → protein). The “retro” part of the family name derives from this step. After reverse transcription, the viral DNA is integrated into the host genome. This DNA is then transcribed and translated normally, giving rise to the viral proteins and also the RNA genome that will be packaged into progeny virions. The integration of the viral DNA into the host’s genome allows retroviruses to maintain a persistent infection, as their integrated DNA is replicated along with the host’s by the cellular replication machinery. The reverse transcription process and subsequent integration step are the defining life cycle features that distinguish the *Retroviridae* from other viruses.

All retroviruses share a basic structure when visualized by electron microscopy. There is an internal electron-dense complex, or core, whose shape varies between retroviral genera. But for all types of retrovirus, this core is surrounded by a lipid bilayer membrane, from which envelope glycoproteins protrude as spikes on the virion surface [for review, (67)]. There are seven genera of *Retroviridae*. These seven fall into two categories, simple and complex, and this division is based on the viral genome. Simple retroviruses encode only four genes (which may be thought of as the “minimal” genes required for replication). These genes are *gag*, *pol*, *pro*, and *env*. They may share reading frames or be expressed in separate ORFs. Complex retroviruses encode the four genes mentioned above as well as accessory genes with diverse functions. The simple retroviruses are alpha-, beta-, and gammaretroviruses. Alpharetroviruses

contain a centrally-placed spherical core (example: avian leukemia virus). Betaretroviruses contain a core which is either cylindrical or round and is eccentrically placed within the virion sphere (examples: mouse mammary tumor virus, Mason-Pfizer monkey virus). Gammaretroviruses also contain a central spherical core (example: leukemia viruses, e.g. murine and feline). Complex retroviruses are delta-, epsilon-, lenti- and spumaviruses. The deltaretroviruses contain a spherical core and accessory genes *tax* and *rex* (example: human T-lymphotropic viruses [HTLVs]). Epsilonretroviruses also have a spherical core and 1-3 additional open reading frames (*A*, *B*, *C*). The only examples of this type of retrovirus are found in fish (e.g. wall-eye dermal sarcoma virus) and reptiles. The lentiviruses have unique core morphology – the core complex from immature virions is spherical, but upon maturation it becomes cone-shaped or cylindrical. Lentiviruses also express several accessory genes. Examples are *vif*, *vpu*, *vpr*, *tat*, *rev* and *nef* from HIV-1, and *tat*, *rev* and *S2* from equine infectious anemia virus (EIAV). Finally, spumaviruses contain a central, uncondensed core which does not exhibit morphology changes upon maturation (example: human foamy virus). Spumaviruses encode two accessory genes, *bel-1* and *bet*. [For review (45, 54-56, 67, 191)]

Although the different genera of *Retroviridae* exhibit varying core morphology and accessory genes, all encode the same four basic genes and therefore show a similar overall virion structure. *Gag* encodes the structural proteins capsid (CA), matrix (MA), nucleocapsid (NC) and proteins of various sizes typically containing “late domains”. All retroviruses have an RNA genome, which is enclosed in the core complex and is itself associated with NC [for review (67)]. Surrounding the RNA/NC complex is the core complex, which is composed mostly of CA (86, 111, 143, 171, 197). This core is surrounded by MA, which is in turn surrounded by the lipid bilayer membrane derived from the host cell (11, 96, 172). Within the lipid bilayer are the

protein products of *env*: the envelope glycoproteins surface unit (SU) and transmembrane (TM). TM passes through the lipid bilayer and anchors SU on the virion surface (57, 181). Also located within the core complex are those proteins derived from *pol* and *pro*: reverse transcriptase (RT), integrase (IN) and protease (PR) (113, 163).

Retroviruses go through a complex series of steps during their infection cycles. Though some details may differ, overall the steps are the same for all *Retroviridae*. They are divided into two parts: early and late stages. The early stage describes the part of the life cycle between the virion and provirus, and the late stage describes the part of the cycle between provirus and infectious virion. The life cycle steps are briefly summarized below.

**Entry: receptor binding/membrane fusion.** All retroviruses require that the viral envelope glycoprotein trimer interact with one or more cell surface molecules, which are known as receptors. This binding leads to conformational changes within the envelope glycoprotein complex, which brings viral and target cell lipid membranes closer together and allows membrane fusion. Upon fusion, the virion core complex is internalized into the cell cytoplasm. [For review (16, 38, 155, 203)]

**Uncoating.** The term “uncoating” refers to events that happen after the viral core has entered the cytoplasm, prior to reverse transcription. These events are still poorly understood. During this stage, it is expected that the viral core will disassemble to some degree. However, it is still not clear whether reverse transcription of the RNA genome requires specific disassembly events to happen before it can initiate. Viruses defective in this step may manifest defects in creating reverse transcripts. Many mutations that appear to interfere with uncoating map to the viral capsid protein. [For review (9, 161, 206)]

***Reverse transcription.*** The viral RNA genome is converted to double-stranded DNA at this step, which takes place shortly after the core complex enters the cytoplasm. Reverse transcription itself takes place within a large complex that contains the RNA, reverse transcriptase, NC, IN and possibly CA (19). Although reverse transcription appears to be contingent upon the viral core being exposed to high levels of dNTPs in the cytoplasm, some viruses may initiate it much earlier, during assembly or release of the particle (141, 156, 208, 218, 219, 227). Reverse transcription follows an order of discrete steps: formation of minus-strand strong-stop DNA, first translocation, long minus-strand synthesis, initiation of plus-strand synthesis, removal of the tRNA primer, second translocation, and completion of both strands. [For review (10, 45, 83)]

***Integration.*** The product of reverse transcription is a full-length double-stranded linear DNA complex, flanked at each end by long terminal repeat sequences (LTRs). After reverse transcription, this complex enters the nucleus. The actual integration of viral DNA is mediated by IN. IN processes the DNA ends and then catalyzes a strand transfer reaction in which the recessed ends make breaks in the target host DNA (183). The resulting intermediate is repaired by cellular DNA repair machinery. The protein complex involved in integration of the provirus is called the pre-integration complex (PIC). Viral proteins associated with the PIC may include CA, RT, IN, MA and NC. [For review (61, 135, 167, 211)]

***Expression of viral RNAs, translation and protein processing.*** The integration of the viral DNA marks the end of the early stage of infection. The late phase begins with the synthesis of viral RNAs. The first product is a long primary transcript. This transcript may be spliced to form a number of subgenomic transcripts that will be translated into protein, or it may remain unspliced; the unspliced transcript can be translated or may be packaged as the RNA genome of

progeny virus. [For review (5, 43, 95, 117, 154)] The *gag*, *pol*, and *pro* genes are translated as long polyprotein precursors (e.g. HIV-1 Gag precursor Pr55). These polyproteins are processed by protease (PR) during or after assembly to form the mature virion particle (e.g. Gag Pr55 → MA, CA, NC and p6). In all retroviruses, *env* is expressed from a subgenomic mRNA, but *gag*, *pol* and *pro* gene arrangement differs. *Pro* may be fused in-frame to the end of *gag* or the beginning of *pol*, or it may have its own ORF. In addition, *gag* and *pol* may be in the same ORF (epsilon-, gammaretroviruses) or may be in different ORFs and require a frameshift during translation (alpharetro-, lentiviruses). In addition, the complex retroviruses express a number of small accessory proteins translated from subgenomic mRNAs. [For review (31, 68, 85, 90, 91, 103, 213)]

***Virion assembly/RNA packaging.*** As viral proteins are synthesized, they begin to assemble to form the immature particle. This process is mainly driven by the uncleaved Gag precursor. Assembly may occur either at the plasma membrane or in the cytoplasm followed by subsequent transfer to the plasma membrane. Proteins derived from *pol* and *env* as well as accessory proteins are incorporated into the assembling virion through contacts with the Gag polyprotein. In addition, the envelope glycoprotein spikes are concentrated at the sites from which the particles will bud. Some host proteins may also get packaged into particles, such as cytoskeletal proteins or various host factors (e.g. HIV-1 and cyclophilin A). The RNA genome is incorporated via the interaction of specific nucleotide sequences in the RNA (Psi  $\psi$  sequences) with the nucleocapsid (NC) region of the Gag polyprotein. [For review of assembly, (1, 79, 85, 149). For review of RNA packaging, (17, 108, 178)]

***Protein processing/virion maturation.*** Upon budding, the precursor proteins must be cleaved to give rise to a mature particle. This cleavage is carried out by PR, which first cleaves itself out of its precursor and then cleaves the other polyproteins. The Gag precursor is cleaved into MA, which forms a shell within the inner face of the membrane; CA, which condenses to form the distinctively-shaped virion core; NC, which coats the RNA genome; and, in lentiviruses, a fourth protein which may vary in size (e.g. HIV-1 and p6, EIAV and p9). The Pol precursor is cleaved to yield RT and IN, and the Env precursor to SU and TM subunits. The morphological change from an immature particle (spherical core with an electron-lucent center) to a mature particle (condensed electron-dense core structure with a distinctive shape) is particularly reliant upon the processing of the Gag polyprotein. Upon maturation, the virion is ready to re-initiate the infection cycle. [For review: (2, 14, 29, 51, 85, 148, 179)]

Due to their integration into the host genome, retroviruses are not easily eliminated by the host's immune system. The host remains infected for life and may have chronic low-level viremia. The effects of retroviral infection vary widely by virus. Since the provirus inserts into the host genome, each insertion event represents a mutation. Over time, accumulation of these mutations can affect genes involved in cell growth and division, resulting in abnormal cell proliferation. Many of the simple retroviruses cause disease in this manner, leading to tumor formation or what are termed "slow" leukemias [for examples, see (47, 63)]. In addition, some retroviruses may encode genes that cause rapid tumor formation, which they have picked up via recombination events with the host genome. These genes are then expressed at high levels in the infected cell. This type of retrovirus is called an acute transforming virus due to its ability to rapidly transform cells and initiate tumorigenesis (189, 202). Finally, there are retroviruses that are directly cytopathic. This group includes many of the better-known complex retroviruses,

including those which infect humans, such as the lentivirus HIV-1. Aside from replication-competent retroviruses and acute transforming retroviruses, all cells contain retrovirus-like elements integrated into the host genome, termed endogenous retroviral elements. These elements have accumulated over time, cannot be removed, are rarely lost, and are usually heavily methylated and not transcribed. In addition, the endogenous retroviral DNA sequence is usually not capable of encoding proteins due to numerous stop codons and frameshifts within ORFs. [For review (142, 169)] In addition, some of these retroelements can transpose intracellularly. Such a transposition event essentially creates a “new” provirus, insertion of which constitutes a new mutation within the host’s genome. [For review (54, 55)]

The study of the retrovirus life cycle has led to the development of retroviruses and retroviral-based vectors as gene transfer tools. Particles can be generated in a packaging cell line that includes *gag*, *pol* and *env* genes but which packages the gene to be transferred rather than the viral RNA genome (6, 38, 46, 144-146). The resulting virus infects cells and the transferred gene integrates into the host genome. Because retroviral particles can easily be pseudotyped with the envelope proteins of other viruses, it is easy to target these recombinant viruses to a specific cell type or multiple cell types (60, 193, 214). Integration into the host’s DNA ensures that the gene of interest will not be lost upon cell division.

## **Lentiviruses**

The best-known retroviruses are those that cause disease in humans, particularly the lentivirus HIV-1. Although lentiviruses share the same basic infection cycle, genes, proteins and virion morphology as other retroviruses, there are some important differences particular to this genus. The most observable difference involves core morphology: although lentiviruses have spherical

cores upon budding, the mature virion has a cone-shaped core (23, 32, 59, 157). In addition, lentiviruses commonly encode the regulatory proteins Tat and Rev in addition to the typical proteins encoded by *gag*, *pol*, *pro* and *env*. Tat (Transactivator of transcription) is involved in driving transcription from the LTR. Basal levels of transcription are usually low. Tat interacts with the LTR via a sequence region called the transactivation response element (TAR). Tat then recruits various transcriptional complexes to the LTR, such as the positive transcription elongation factor b (pTEFb) complex; this increases the elongation activity of the RNA polymerase II complex. [For review (42, 71, 115, 184, 185)]

The Rev protein is involved in the translocation of unspliced or partially-spliced mRNA from the nucleus to the cytoplasm for translation or virion encapsidation. Lentiviral genomes are relatively small, but must express a variety of proteins. This is accomplished via alternative mRNA splicing, which results in the production of various mRNAs from a single RNA precursor. Three types of mRNA are transcribed: a full-length unspliced mRNA that will be packaged as the viral genome; singly-spliced mRNA encoding the major protein precursors; small multi-spliced transcripts. Most cellular mRNAs are fully spliced before nuclear export, but lentiviruses by necessity must export unspliced or singly-spliced transcripts. This is accomplished through action of the Rev protein. Rev binds to the Rev-response element (RRE) located near the end of the *env* gene and mediates mRNA transport to the cytoplasm. Aside from these two accessory proteins, lentiviruses may express other regulatory proteins. [For review (36, 98, 99, 129, 186)]

Another key lentivirus feature is their ability to infect nondividing cells. It is this ability that allows lentiviruses such as HIV-1 and EIAV to infect their target cells, which include



quiescent T cells for HIV-1 and macrophages for both viruses (127, 128, 221). The PIC reaches the nucleus through the nuclear pore complex. Because of the size of the PIC, entry into the nucleus must require active transport rather than passive diffusion through the NPC (28, 49, 88, 130, 212). The translocation of the HIV-1 PIC into the nucleus has been a field of extensive study, and researchers have sought to identify viral components critical for this process as well as the cellular machinery involved. This process will be discussed in further detail below.

### **Equine infectious anemia virus (EIAV)**

HIV-1 is the best-known lentivirus, but a number of other lentiviruses also induce long-term persistent infections that cause diseases which can have serious health ramifications for the host. One such virus is EIAV. EIAV is a member of the non-primate lentivirus group, which also includes visna/maedi virus (VMV), caprine arthritis-encephalitis virus (CAEV) and feline and bovine immunodeficiency viruses (FIV and BIV). EIAV was the first retrovirus associated with disease, being identified as a causative “filterable agent” in 1904. EIAV is transmitted through blood exposure, either through unsterile needles/transfusions from infected carriers, or through insect vectors (69, 70). The most common method of transmission is through bites from horse flies or other hematophagous insects. The virus is carried on the insect’s mouth parts after a blood meal from an infected carrier (44, 94, 101). The pathogenesis of EIAV infection is unique. The course of the disease can be divided into three distinct phases. The acute phase occurs shortly after infection and is marked by fever and anemia. These symptoms are usually mild and few animals succumb to the disease at this point (126, 153). The next phase is called the chronic phase. This stage lasts 1-12 months and is characterized by recurrent cycles of severe anemia and high titers of circulating virions (102, 126, 153, 164). If the infected animal survives the

chronic phase, it enters the asymptomatic phase. In contrast to other lentiviruses such as HIV-1, in which carriers slowly succumb to degenerative diseases within a few years of infection, EIAV-infected horses transition from the chronic phase to a stage marked by a lack of clinical symptoms. However, carriers remain infected for life and can still transmit the virus through blood exposure. During the asymptomatic phase, viremia is extremely low (37, 101, 116, 126, 180, 190). This phase is reversible, and animals may re-enter the chronic phase and recurrent bouts of anemia. Immune response is clearly critical for maintenance of the asymptomatic phase, as administration of immunosuppressive drugs leads to disease recrudescence (41, 119, 174, 209).

EIAV undergoes the life cycle steps typical of all retroviruses and exhibits characteristic lentiviral structure. However, it does have some unique features that differentiate it from other lentiviruses, which will be summarized below.

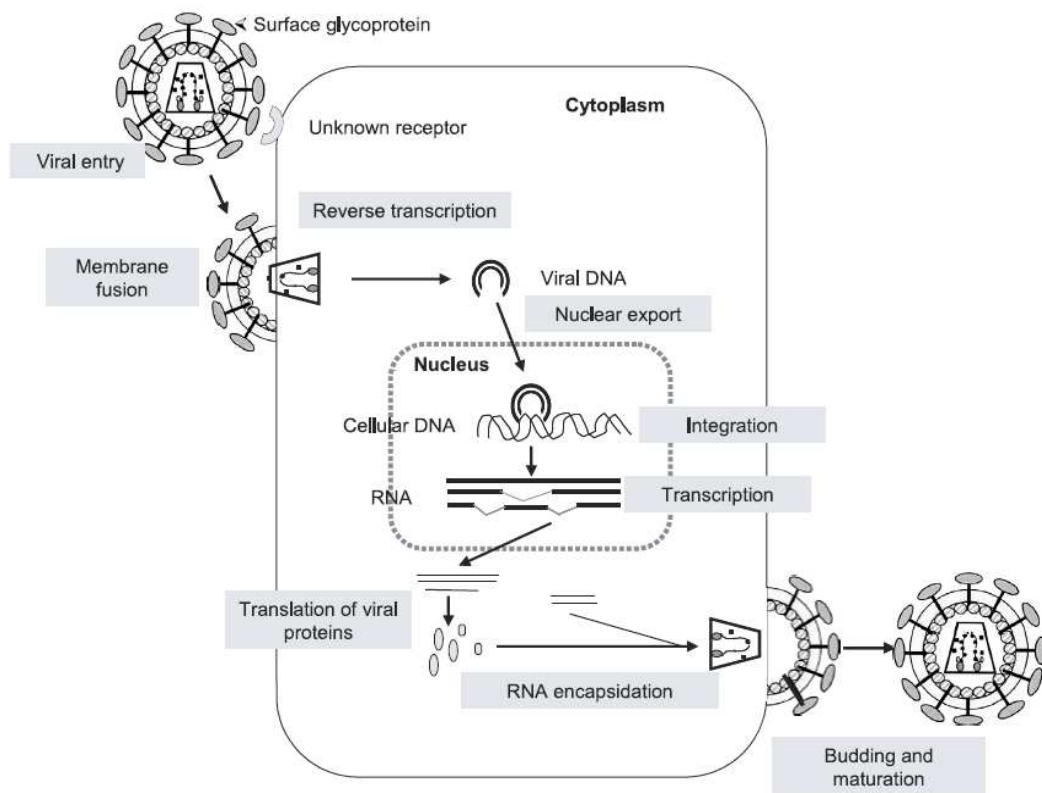


Figure 1-1. The infection cycle of EIAV and other retroviruses.  
 From Leroux *et al.* EIAV: what has HIV's country cousin got to tell us? Vet. Res. 35 (2004) 485-512

***EIAV entry and the cellular receptor.*** EIAV is an exclusively macrophage-tropic virus. This particular type of tropism is shared by VMV and CAEV, which infect sheep and goats and are referred to as small ruminant lentiviruses (SRLVs). SRLVs infect monocytes and macrophages. Infection of monocytes requires their subsequent maturation to macrophages for active virus replication (158, 159, 168, 195). EIAV infects circulating blood macrophages which then disseminate the virus to mononuclear phagocytes in various organs. The main sites of virus replication are macrophages in the liver and spleen (168).

The viral envelope SU region (gp90) is predicted to be similar to that of HIV-1 (73, 74). This region is the predominant site of antigenic variation (127, 128, 225). A cellular receptor for

EIAV has been identified fairly recently. Equine lentivirus receptor-1 (ELR1) is a member of the tumor necrosis factor receptor (TNFR) superfamily. Other retroviruses, including FIV and avian sarcoma-leukosis virus (ASLV), also use TNFR proteins as receptors (3, 25). ELR1 is sufficient for EIAV entry; no coreceptors are needed, in contrast to HIV-1 and other lentiviruses. In addition, ELR1-mediated entry of EIAV is pH-dependent (106, 221). A complex of a proteoglycan and a protein kinase has been identified as a functional receptor for VMV, suggesting that ungulate lentiviruses with exclusive macrophage tropism such as EIAV and VMV may have a common entry mechanism in which a single receptor protein mediates pH-dependent endocytosis (12, 27, 106). In addition, it is hypothesized that those lentiviruses with monocyte/macrophage tropism, like the ungulate lentiviruses, have evolved to use receptors that will activate normally quiescent target cells, leading to enhanced gene expression and virus replication in these nondividing cells. TNFR proteins activate diverse signal transduction pathways and the VMV receptor complex is associated with activation of mitogen-activated protein kinase pathways (12, 13, 139, 192). Current research is ongoing to determine the cellular expression profile of the ELR1 receptor *in vivo*. NIH 3T3 cells expressing ELR1 and equine cyclin T1 are permissive for EIAV replication. In addition, ELR1 is expressed in all cell types permissive for EIAV infection, including macrophages, equine dermal fibroblasts and fetal equine kidney cells. ELR1 has not been detected in nonpermissive cell types, such as equine lymphocytes, simian or human cell lines (222). In addition, it is not yet known whether ELR1 is the only functional cellular receptor for EIAV.

***EIAV proteins.*** The EIAV genome is 8.2 kb, the smallest and simplest of the lentiviruses (Fig. 1-2B). *Gag* and *pol* genes are translated from the full-length viral RNA (162). The Gag polyprotein (Pr55) is cleaved by protease into four structural proteins: MA (p15), CA (p26), NC

(p11) and p9. EIAV MA is associated with the inner face of the viral membrane and shows many structural similarities with HIV-1 MA. However, HIV-1 MA is myristylated and EIAV MA is not (89). The p9 protein is analogous to late domain proteins such as HIV-1 p6. The p9 protein is involved in particle budding but also plays a role in the nuclear import of proviral DNA (33, 105, 150, 175, 176, 198). *Pol* products include RT, PR, IN, and a dUTPase (138, 194, 204). The dUTPase protein p15 is essential for EIAV replication in macrophages. *Env* encodes gp90 and gp145 (128). Besides *gag*, *pol*, and *env*, EIAV has three additional reading frames that encode the proteins Tat, Rev and S2 from multispliced transcripts (177, 196). Rev mediates the transport of viral mRNAs from the nucleus to the cytoplasm by interacting with an RRE (15, 35, 126, 147). EIAV Tat interacts with the viral LTR through a TAR element to drive transcription. Tat recruits the pTEFb complex, which is composed of CDK9 kinase and cyclin T1. Despite the fact that EIAV Tat and TAR elements are highly divergent from those of HIV-1, a similar process involving cyclin T1 is involved in HIV-1 gene expression (4, 18, 22, 126, 137, 200). Murine NIH 3T3 cells engineered to express the receptor ELR1 and equine cyclin T1 are permissive for EIAV replication, indicating that these two factors are sufficient for infection and murine cells do not contain negative host factors that block infection (222). EIAV is unique among lentiviruses in having only one accessory gene besides *tat* and *rev*. This is the *S2* gene. The *S2* protein is not required *in vitro* for virion production and is not packaged into virions. However, it is present in all EIAV strains and is required for infection *in vivo*. Currently, it is not known what the exact function of *S2* is, although a yeast two-hybrid screen has identified several cellular proteins that interact with *S2*. One possible function to date is to increase the gene expression of pro-inflammatory cytokines and chemokines (39, 40, 62, 132, 133, 170, 188).

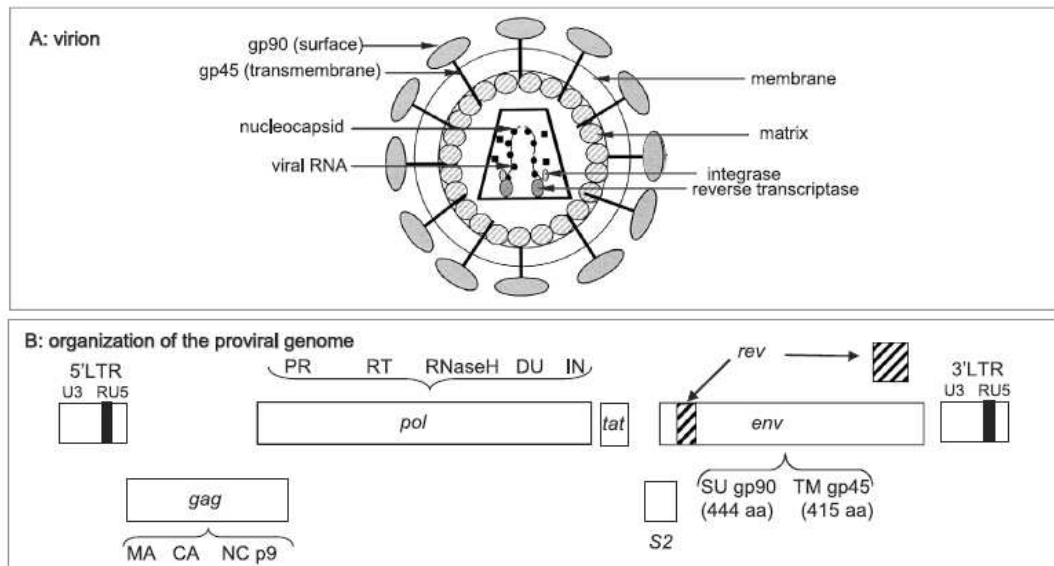
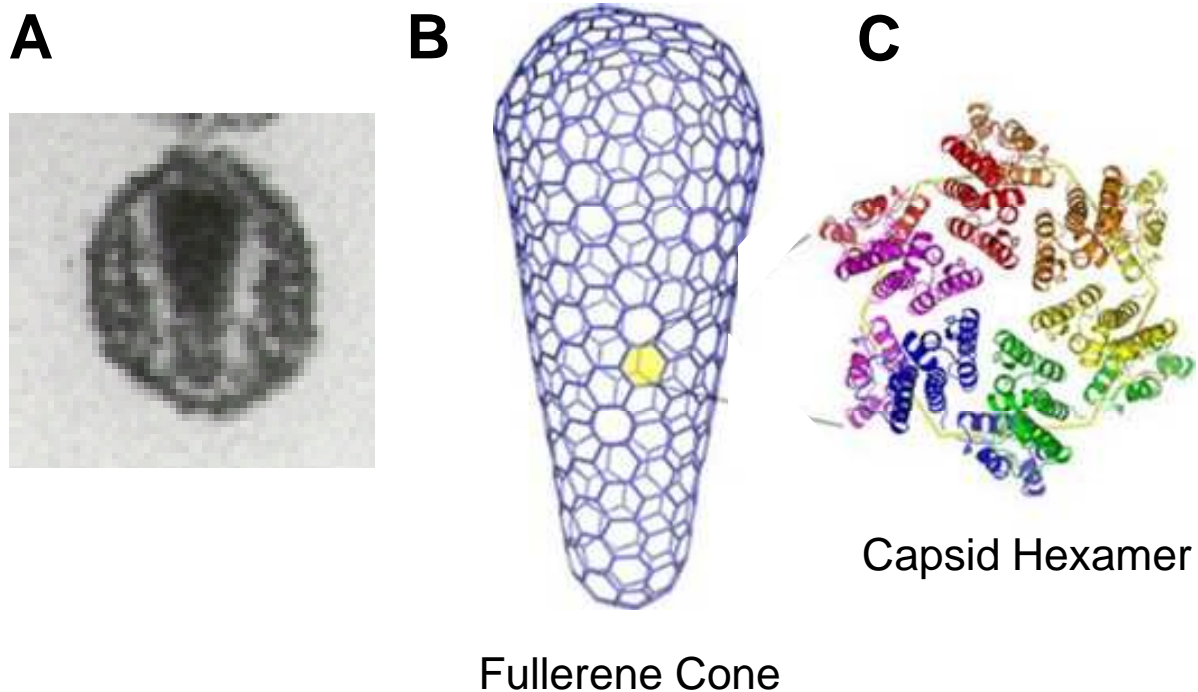


Figure 1-2. The EIAV virion (A) and proviral genome (B).  
 From Leroux *et al.* *EIAV: what has HIV's country cousin got to tell us?* Vet. Res. 35 (2004) 485-512

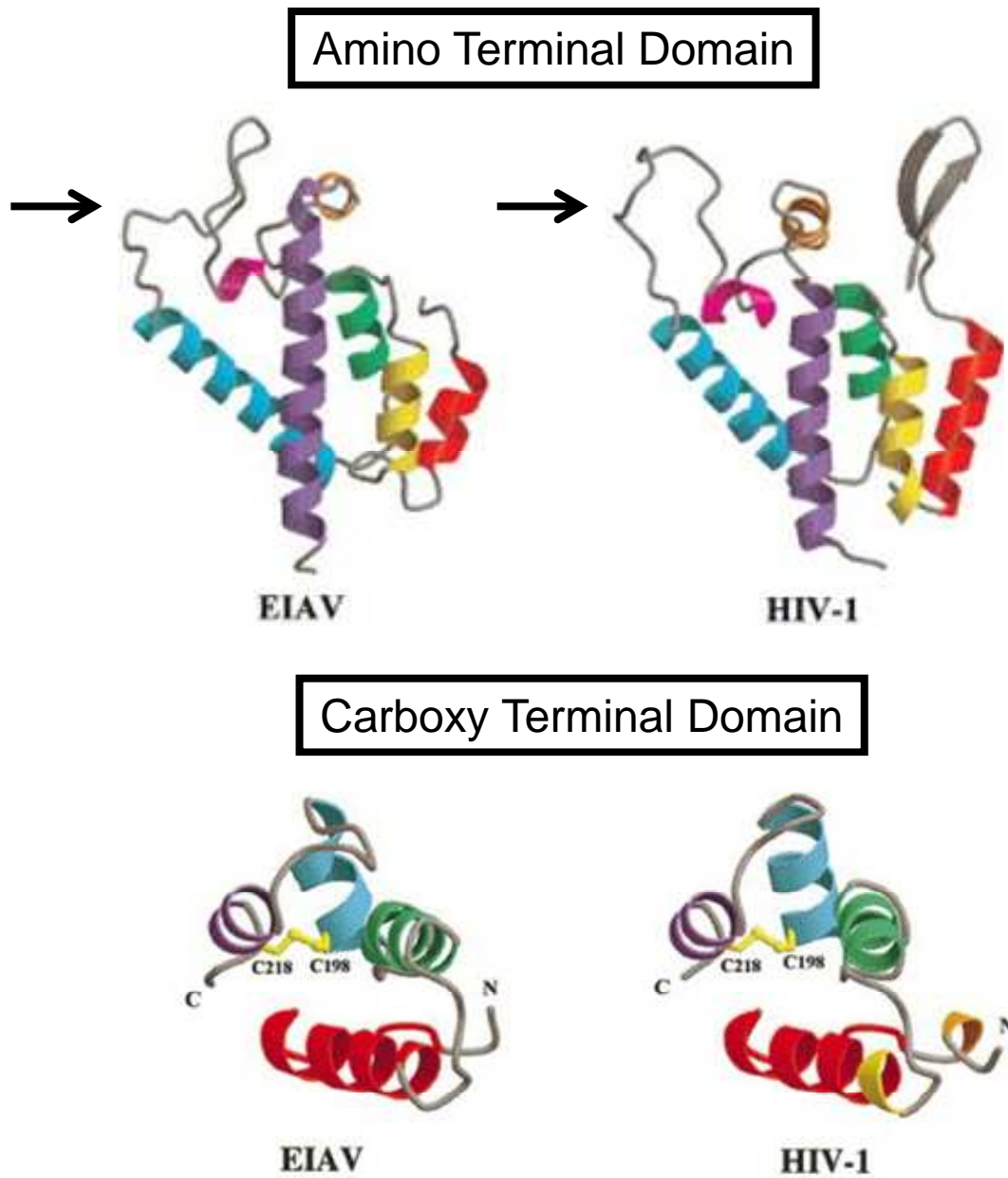
**EIAV capsid (CA).** The EIAV core complex within the lipid membrane of the virion is mostly composed of CA protein (p26). When virions are viewed by thin-section electron microscopy, the characteristic cone shape of the inner viral core is easily observed (Fig. 1-3A). This type of structure is called a fullerene cone: a variably curved shell composed of hexamers and pentamers of CA (Fig. 1-3B) (81). The EIAV viral core complex exhibits the typical cone-shaped morphology that is the hallmark of lentiviruses. Like HIV-1 CA, EIAV CA forms dimers in solution. However, in mature lentiviral virions, CA condenses to form a fullerene cone: a hexagonal lattice of CA monomers which are closed by pentons [reviewed (80)]. Each hexagonal unit of the fullerene cone represents a hexameric ring of six CA monomers (Fig. 1-3C) (24, 78, 124, 134, 160). These fullerene cone structures can be purified from detergent-treated EIAV virions and retain their cone shape under electron microscopy [(182), Appendix].

The simple purification of these complexes and their ability to retain their three-dimensional state after isolation suggests that EIAV cores may be unusually stable.



**Figure 1-3.** The lentiviral core complex is a fullerene cone composed of CA hexamers. (A) An electron micrograph of a HIV-1 virion. (B) The structure of a fullerene cone. A single CA hexamer is highlighted in yellow. (C) The structure of an individual CA hexamer, with each of the six CA monomers portrayed in a different color.

The structure of EIAV CA has been solved (107). EIAV CA has 30% sequence identity with HIV-1 CA (87). Like HIV-1 CA, EIAV CA consists of seven  $\alpha$ -helices forming the N-terminal domain (NTD) and four  $\alpha$ -helices forming the carboxyl-terminal domain (CTD), with a linker region between the two (Fig. 1-4). Like HIV-1, EIAV CA has a proline-rich loop between helices 4-5 in the NTD. This loop in HIV-1 CA has 4 prolines in 17 amino acid residues, and assumes both *cis* and *trans* conformations (76, 77, 82, 210). The EIAV proline-rich loop has 7 prolines in 19 residues and adopts only the *trans* conformation in the crystal (107).



**Figure 1-4.** The EIAV and HIV-1 CA share similar domain structures. The proline-rich loop found in the amino-terminal domain of both CA proteins is indicated with black arrows. Figure adapted from (30).

The HIV-1 CA is the target of several cellular host factors that can affect infectivity, primarily during the uncoating step but also during nuclear import. EIAV CA has also been shown to interact with some of these same factors (see below). These similarities between the CA proteins



of these two viruses, along with the ease of isolating intact EIAV core structures, make EIAV an attractive candidate for the study of lentiviral capsids.

**Cellular host factors: uncoating.** The incoming retroviral CA is a target for cellular host factors that modulate the uncoating process. Host factors may influence uncoating either positively or negatively. One class of factors that negatively affect uncoating have been called restriction factors (93, 97, 120, 165, 166). The TRIM5 $\alpha$  proteins are an example of restriction factors. It has been shown that TRIM5 $\alpha$  from Old World monkeys such as the rhesus macaque binds the HIV-1 capsid and accelerates the disassembly of the core complex. This leads to a severe block in HIV-1 infection that usually occurs prior to reverse transcription. In addition, human TRIM5 $\alpha$  exhibited similar inhibitory effects on N-MLV infection (52, 53, 173, 199). EIAV infection is severely restricted by rhesus TRIM5 $\alpha$  and restricted to a lesser extent by human TRIM5 $\alpha$  (92, 207).

Cyclophilin A is an example of a host factor that positively modulates uncoating. Cyclophilin A (CypA) interacts with the HIV-1 CA. It is a peptidyl prolyl isomerase whose normal cellular function is catalysis of the *cis/trans* isomerization of proline peptide bonds (84). CypA binds HIV-1 CA via the proline-rich loop located in the NTD of CA. Residues Gly89 and Pro90 are the primary CypA recognition motif (76, 210, 224). Disruption of this binding, whether through amino acid alteration or application of the drug cyclosporine A (CsA), results in decreased HIV-1 infection in certain cell types (76, 109, 114, 136, 217). EIAV CA has a proline-rich loop very similar to that of HIV-1; however, treatment of cells with CsA does not affect infectivity, suggesting that CypA binding is not required for EIAV infectivity. The

proline-rich loop region of EIAV is an attractive candidate for further study, due to this region being critical for infectivity of several other lentiviruses.

***Cellular host factors: nuclear import.*** The early stages of the retroviral infection cycle are often pictured sequentially, with reverse transcription and integration happening after uncoating has already taken place. Both reverse transcription and integration involve large nucleoprotein complexes. Reverse transcriptase converts viral RNA into double-stranded DNA in the reverse transcription complex (RTC) (65, 66). Viral DNA enters the nucleus in the form of the PIC, at which time it is integrated into the host genome (19, 26, 72). Initially, it was believed that uncoating must be complete before reverse transcription could begin, as very little CA remains associated with the RTC (65, 66, 100). However, HIV-1 is able to initiate reverse transcription within the intact core complex *in vitro* (223).

Unlike simple retroviruses, lentiviruses are able to infect non-dividing cells, such as the macrophage, the host cell type of EIAV. Therefore, these viruses must be able to cross the nuclear envelope that separates nucleus and cytoplasm (201). Lentiviruses are believed to enter the nucleus through the nuclear pore complex (NPC) (112, 131). Molecules can move through the NPC either by passive diffusion or active transport. As the PIC is too large to enter by diffusion (> 9 nm limit), it must be actively transported by the cellular import machinery (28, 49, 88, 130, 152, 212). Various studies have identified several components of NPC transport machinery involved in HIV-1 PIC nuclear entry, including nucleoporins (NUPs), importins and karyopherins (8, 58, 75, 220). More recently, siRNA screens have identified critical transport proteins by examining the effects of depletion of said proteins on HIV-1 infection (21, 118, 226). One such protein identified was transportin 3 (TNPO3). TNPO3 is a member of the karyopherin

$\beta$  superfamily and transports proteins with serine/arginine motifs into the nucleus (110, 122, 123). Additional proteins identified were NPC components NUP153, NUP358 and NUP155 (21, 118). On the viral side of nuclear transport, several HIV-1 proteins have been assigned roles as well. These include MA, Vpr and IN as well as the DNA flap formed during reverse transcription (104). However, a definitive role for these proteins has not been established, and viruses with these proteins mutated in combination could still infect nondividing cells (50, 64). Yamashita *et al.* have shown that the definitive requirement for nuclear import of the PIC is instead CA (215, 216). This suggests that CA is required beyond the step of core disassembly and that the minimal amounts of CA that remain associated with the nucleoprotein complexes post-uncoating play a critical role in subsequent early infectivity steps.

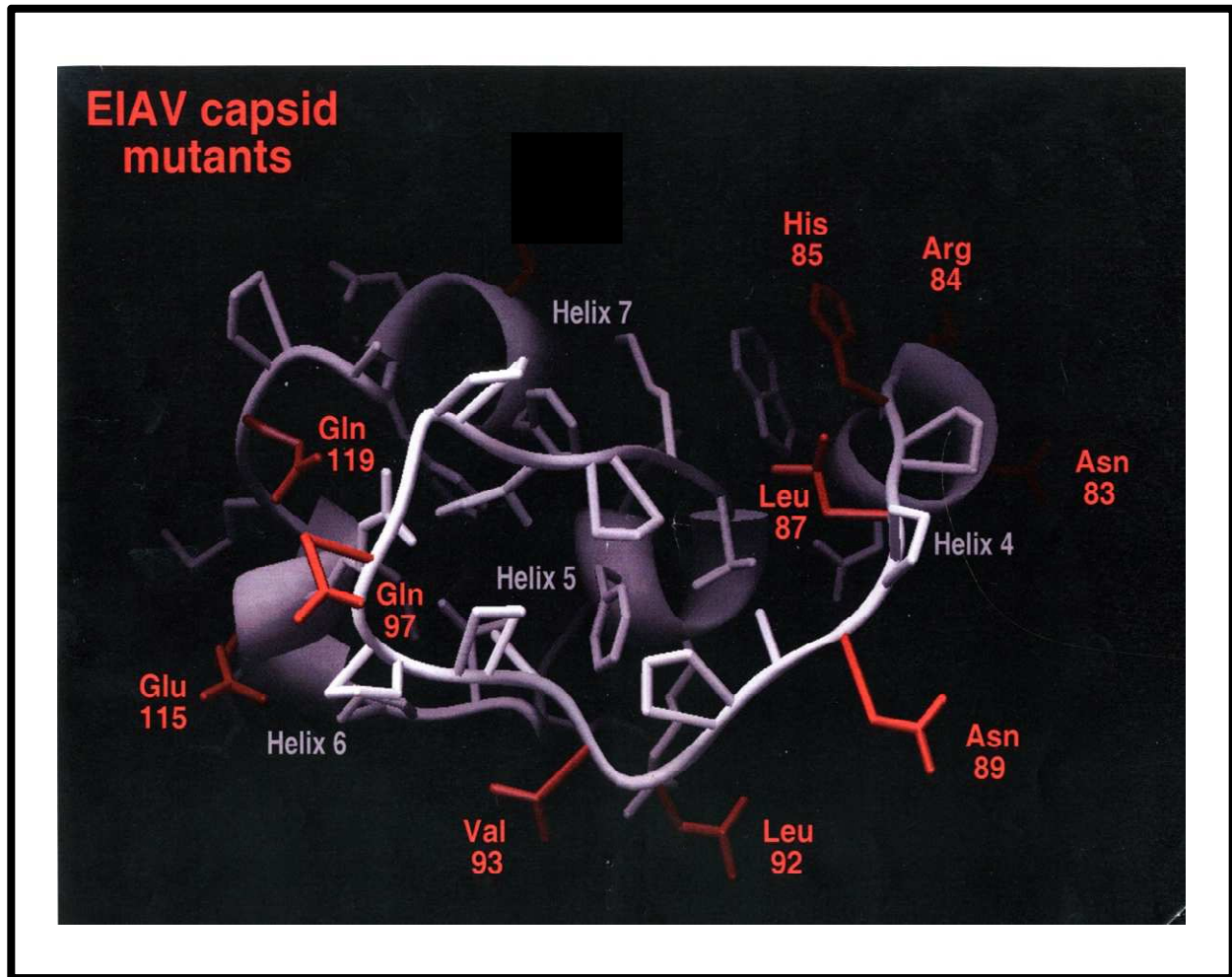
Because TNPO3 was identified as an IN binding factor in a yeast two-hybrid screen, it was initially thought that the requirement of HIV-1 for TNPO3 in nuclear import would map to IN (34). However, it mapped instead to CA. CA mutant N74D does not require TNPO3 for infection (7, 48, 121, 125, 140, 187, 205). In addition, the requirement for NUP153 is determined by CA as well. Matreyek *et al.* demonstrated that chimeric HIV-1 viruses containing CA from MLV were insensitive to NUP153 depletion (151).

To date, most studies of nuclear import/CA requirements have focused on HIV-1. However, the interaction of HIV-1 with CypA has complicated interpretation of some data. CsA treatment renders wild-type HIV-1 insensitive to NUP153 knockdown (151). However, SIVmac is highly sensitive to NUP153 knockdown despite not binding CypA (20, 151). In addition, the HIV-1 CA mutant N74D is insensitive to TNPO3 knockdown but is more sensitive to CsA treatment and is also unable to infect macrophages (7). Aside from CypA's effects on nuclear

import, Li *et al.* have demonstrated that the CypA/CA interaction can either stabilize or destabilize the incoming HIV-1 CA, depending on the target cell type (136). EIAV has a loop region similar to HIV-1's CypA-binding loop but does not bind CypA. Therefore, experiments involving EIAV CA and nuclear import should be easier to interpret without confounding results due to CypA. A study investigating the TNPO3 domain required for virus import has shown that EIAV is extremely sensitive to TNPO3 depletion and that the same TNPO3 motifs (the cargo-binding domain) are required for HIV-1 and EIAV infection (140).

Recent studies have shown that the role of CA does not end at the uncoating step. Rather, CA remains a critical infectivity factor throughout the early stages of infection up to integration of the provirus. Due to its similarities to HIV-1 CA and its purported stability, we postulated that the EIAV CA would be an attractive target for mutagenesis. As both HIV-1 and EIAV contain the amino-terminal proline-rich loop, and changes to this loop affect HIV-1 uncoating, we expected that the introduction of changes in this region would affect infectivity during the uncoating step. We aligned the loop regions of HIV-1 and EIAV and introduced amino acid substitutions at conserved residues, excluding the prolines (Fig. 1-5). Chapter 2 contains the results of experiments on these mutants. Briefly, we saw that some changes did affect infectivity, in both negative and positive directions. For those mutants that had deleterious effects, infectivity was abrogated to a severe degree. Unexpectedly, the changes did not affect uncoating or reverse transcription. However, those mutants with infectivity defects exhibited markedly decreased production of 2-LTR circles and proviral DNA. This suggests that these particular mutants have defects in nuclear import, demonstrating that EIAV CA should be investigated further as a target for the nuclear import process. These mutants in particular may

be useful tools for future research investigating the involvement of CA in the nuclear import of lentiviruses.



**Figure 1-5.** Amino acid substitutions introduced into the EIAV CA proline-rich loop. The loop is highlighted in pale gray. Amino acids chosen for substitution are indicated in red.

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## CHAPTER 2

# RESULTS

Retroviral Gag proteins have critical roles in the infection of target cells during early post-entry events. In particular, the retroviral capsid protein (CA) has been implicated in many early infectivity steps. Viruses must disassemble properly after target cell entry. Several studies have shown that CA is critical to this step, which is termed “uncoating” (13, 18). Mature lentiviruses contain a distinct cone-shaped core comprised of CA monomers. CA monomers interact at several molecular interfaces to form the conical core, including C-terminal domains involved in dimerization and N-terminal domains involved in hexamerization (15, 35, 46). This core complex begins uncoating soon after the fusion of viral and cell membranes (13, 19). Very little CA protein can be found associated with the reverse transcription complex (11, 12, 23). Changes in CA that affect the stability of this core complex can severely abrogate infectivity (13). In addition, the incoming viral CA is the target of several host factors that can affect infectivity. The host cytosolic protein cyclophilin A (CypA) binds to the proline-rich loop in the N-terminal domain of HIV-1 CA (14, 15, 17, 42, 61). Changes in this region that affect CypA binding to CA can be severely detrimental to core disassembly and infectivity (4, 39, 59, 63, 70). Certain host restriction factors target CA and interrupt retroviral infection at the uncoating step. The proteins TRIM5 $\alpha$  and TRIMCyp bind the incoming capsid of certain retroviruses and accelerate core disassembly (6, 8, 47, 55, 60). Changes in CA residues critical for these interactions can allow the incoming viral capsid to escape TRIM5 $\alpha$ -mediated disassembly or cause previously nonrestricted retroviruses to become TRIM5 $\alpha$  targets (36, 37, 40).

More recent studies have shown that CA’s role in the infection process does not end at uncoating. Reverse transcription and subsequent viral cDNA integration take place within nucleoprotein complexes that may retain components of the viral core complex (3, 11, 12, 23,

45). Although certain retroviruses require the dissolution of the nuclear membrane during mitosis, lentiviruses such as HIV-1 or equine infectious anemia virus (EIAV) can infect nondividing cells and therefore are believed to cross the nuclear membrane by passing through the nuclear pore complex (27, 33, 54). The ability of HIV-1 to infect growth-arrested cells has been mapped to CA (67-69). Several recent studies have investigated the requirements for various nuclear import factors during infection, such as nucleoporin Nup153 or transportin 3 (TNPO3). The requirements for these factors map to HIV-1 CA and not to the integrase protein as previously thought (1, 5, 29, 30, 41, 44, 56, 62, 72).

In this study we investigated the effects of changes in the EIAV CA on virus infection. EIAV is an attractive candidate for capsid protein studies as it is a lentivirus like HIV-1 and also contains an N-terminal proline-rich loop that is very similar to HIV-1's Cyp A-binding loop (26). Changes in this loop exert various effects on HIV-1 infection, in some cases dependent on the level of CypA expression in the host cell. EIAV infection is not apparently influenced by CypA. Nonetheless, because of the presumed exposure of this loop on the EIAV capsid, we expected that this region of the EIAV CA would be critical for infection and that changes in this region would yield interesting phenotypes. Our findings show that alteration of the loop region did in fact affect EIAV infectivity. We identified three mutants that demonstrate EIAV CA is also critical for nuclear import. The requirement of CA for proper nuclear import and subsequent proviral integration in both EIAV and HIV-1 highlights the universality of lentivirus CA as a nuclear transport/entry determinant.



## **Materials & Methods**

### **Production of recombinant EIAV expressing green fluorescent protein (GFP).**

The pEV53 plasmid encoding the EIAV Gag/Pol polyprotein and the pSIN6.1CeGFPW plasmid encoding enhanced green fluorescent protein (eGFP) have been described previously (48-51). Point mutations were introduced into the region of pEV53 encoding CA using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). Vesicular stomatitis virus protein G (VSV-G)-pseudotyped EIAV-GFP virions were made by cotransfecting 293T cells with wild-type (wt) or CA mutant pEV53 vector, pSIN6.1CeGFPW vector, and a pHCMV plasmid expressing VSV-G. Lipofectamine 2000 transfection reagent was used (Invitrogen). Virus-containing supernatant was harvested 48 h post-transfection. All virus-containing supernatants were cleared of debris by low-speed centrifugation and then concentrated by centrifugation in a Beckman SW 28 rotor at 27000 rpm for 2 h. Each virus stock was quantitated by a standard reverse transcriptase (RT) activity assay (25). Stocks were stored in aliquots at  $-80^{\circ}\text{C}$ .

### **Cell culture and infectivity assays.**

Human 293T cells (ATCC CRL-11286) and the equine dermal cell line ED (ATCC CCL-57) were acquired from the American Type Culture Collection. Cf2-LPCX cells and Cf2-TRIM5 $\alpha_{Rh}$  cells have been described previously (60). 293T and ED cells were grown at  $37^{\circ}\text{C}$  in Dulbecco modified Eagle medium (Invitrogen). All media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. In addition, media for Cf2-LPCX and Cf2-TRIM5 $\alpha_{Rh}$  cells were supplanted with 5 ng/ml puromycin. For infection, Cf2-LPCX, Cf2-TRIM5 $\alpha_{Rh}$  and ED cells were seeded at a density of  $5 \times 10^5$  cells/well in 6-well plates. Equal amounts of EIAV viruses, normalized by RT activity, were used to infect cells for 4 h at  $37^{\circ}\text{C}$ . Unabsorbed virus was washed out with phosphate-buffered saline (PBS) and the cells were cultured with fresh

media. Cells were harvested 48 h after infection and fixed in PBS containing 4% formaldehyde. The percentage of infected, GFP-positive cells was determined by fluorescence-activated cell sorting (FACS) using FACScan (Becton Dickinson).

#### **Viral protein analysis and Western blotting.**

293T cells were transfected with the pEV3 plasmid encoding either wt. or mutant EIAV Gag/Pol polyprotein. Cells were harvested and lysed 48 h post-transfection. The expression levels of the cell-associated Gag proteins were analyzed by Western blotting with a polyclonal anti-EIAV serum (LADY), gift from the Montelaro lab (Univ. of Pittsburgh), and an anti-horse IgG horseradish peroxidase (HRP)-conjugated antibody (Abcam), respectively. Virions released into the transfected cell supernatants were filtered (0.45- $\mu$ m pore size). Virus-containing supernatant was normalized by volume and the virions lysed. Viral lysates were Western blotted using the same antibody combination described above.

#### **Electron microscopy of virus particles.**

Ten ml of transfected cell supernatant containing wt or mutant virus was centrifuged at 4°C for 2 h at 27,000 rpm in a Beckman SW41 rotor. Pelleted viral particles were fixed with glutaraldehyde and analyzed by thin-section electron microscopy at the Harvard Medical School Electron Microscope Facility.

#### **Quantitative real-time PCR analysis of EIAV-specific viral DNA species.**

Virus stocks derived from transfection of 293T cells were treated with 20 U of DNase I (NEB)/ml for 60 min at room temperature. Approximately  $1 \times 10^5$  Cf2-LPCX cells were infected with 8000 cpm (RT activity) of wt EIAV-GFP virus or mutant EIAV-GFP virus. As a control, cells treated with 150  $\mu$ M azidothymidine (AZT) were infected with wt EIAV-GFP virus. As another control, cells were incubated with wt EIAV-GFP virus lacking envelope

glycoproteins (Env-). Genomic DNA was isolated at various time points after infection using the DNEasy Blood and Tissue Kit (Qiagen), and 250 ng of genomic DNA was used for quantitative PCR amplification. The conditions for quantitative real-time PCR of early and late DNA products have been described previously (25). The primer/probe set used to amplify early DNA products (minus-strand strong-stop DNA) has been described previously (25). The primer/probe set used to detect late DNA products (*eGFP* DNA) was as follows: *eGFP*-FW (5'-CGACGGCAACTACAAGAC -'3), *eGFP*-RV (5'-GTCCTCCTTGAAGTCGATGC-3'), and *eGFP* probe (5'-(6-carboxyfluorescein)-CACCTGGTGAACCGCATCG-(5-carboxytetramethylrhodamine)-3'). For persistent viral DNA (vDNA), the isolated genomic DNA was subjected to quantitative real-time PCR using the *eGFP* primer/probe set. Standard curves for each DNA species were created as described previously (25).

For nuclear unintegrated DNA (2-LTR circles), genomic DNA was extracted as described above and 250 ng of genomic DNA was used for quantitative PCR amplification. The primers used were 2LTR-FW (5'-TGTGCAGACTTGCGAACCATG-3') and 2LTR-RV (5'-CGAATTCCAGCGTCTGAGTTAC-3'). A standard curve was created to calculate the number of copies of 2-LTR circle DNA. The 2LTR primer set was used to amplify a fragment of approximately 860 nt from genomic DNA extracted from cells infected with wt EIAV-GFP (Platinum Blue PCR Supermix, Invitrogen). The resulting fragment was cloned into the pCR 2.1 vector contained in The Original TA Cloning Kit (Invitrogen). The resulting plasmid was used to establish a standard curve, as previously described (25). Quantitative real-time PCR was carried out using the QuantiTect SYBR Green PCR Kit (Qiagen). After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 rounds of amplification were carried out at 95°C for 15 sec, 60°C for 30 sec and 72°C for 1 min 15 sec. Reactions were carried out in the ABI Prism

7700 sequence detection system and analyzed with ABI Prism SDS software (Applied Biosystems).

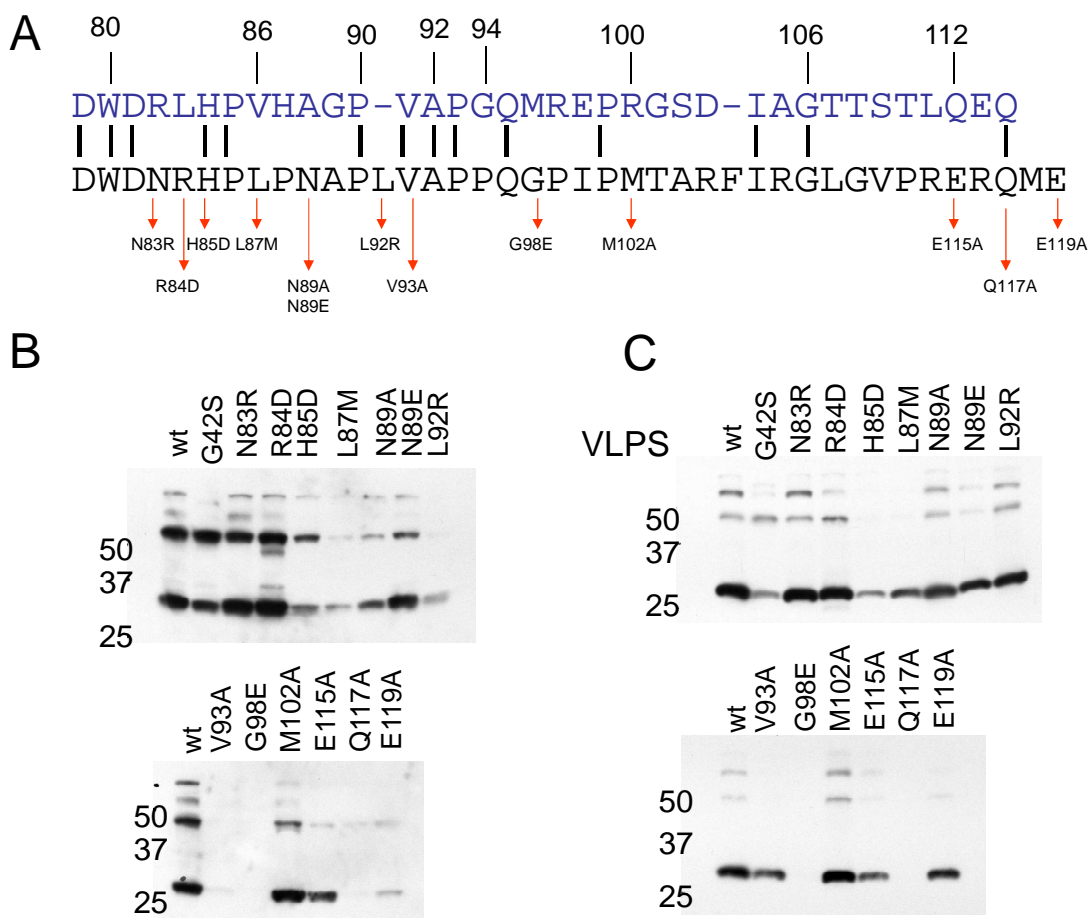
#### **Fate-of-Capsid assay.**

The assay to examine the fate of EIAV capsids in the cytosol of newly-infected Cf2-LPCX cells was performed essentially as described previously (60). Concentrated virus in approximately 100-500  $\mu$ l was incubated with  $5 \times 10^6$  Cf2-LPCX cells in a total of 5 ml of media in a T-75 flask. The cells and virus were initially incubated for 30 min at 4°C and then shifted to 37°C. At 4 h post-infection, the cells were washed with PBS and then incubated with 10 ml of fresh medium for the remainder of the infection. The cells were then washed in ice-cold PBS and lysed in 2.5 ml of hypotonic lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM KCl, 1 mM EDTA) by Dounce homogenization. After the cell debris was removed by centrifugation for 5 min at 2,000 x g, 2 ml of the clarified lysate was layered onto a 7-ml 50%-sucrose cushion and centrifuged at 125,000 x g for 2 h at 4°C. After centrifugation, 100  $\mu$ l of the topmost part of the supernatant was collected. The pellet was resuspended in 120  $\mu$ l of 1X sodium dodecyl sulfate sample buffer. The supernatant and pellet fractions were subjected to Western blotting analysis of CA proteins with an anti-EIAV polyclonal serum.

## **Results**

### **Effects of changes in the proline-rich CA loop on Gag expression, processing, and morphology**

The proline-rich loop (residues 80-119) of the EIAV CA protein protrudes from the helical bundle of the N-terminal domain of CA. Thus, this loop is expected to be exposed on the exterior surface of the assembled CA and corresponds to the CypA-binding loop of HIV-1. To assess the effects of changes in this proline-rich loop region on viral infectivity, we introduced point mutations in the CA-encoding region of a recombinant, single-round EIAV vector (Fig. 2-1A). We examined whether the expression and processing of the Gag polyprotein were affected by these changes. 293T cells were transfected with plasmids encoding either wt or mutant EIAV Gag/Pol proteins. Forty-eight hours after transfection, both cell-associated and virion-associated Gag proteins were analyzed by Western blotting using a polyclonal anti-EIAV serum (Fig. 2-1B and C). Several mutants appeared to be defective, exhibiting either greatly reduced (H85D, L87M, L92R, V93A, E115A) or undetectable steady-state levels of expression (G98E, Q117A, E119A) of Gag polyproteins. In addition, the G42S mutant appeared to exhibit a defect in Gag precursor processing. These results suggest that several of the changes introduced into the CA region affect Gag expression, but not virion assembly and release.



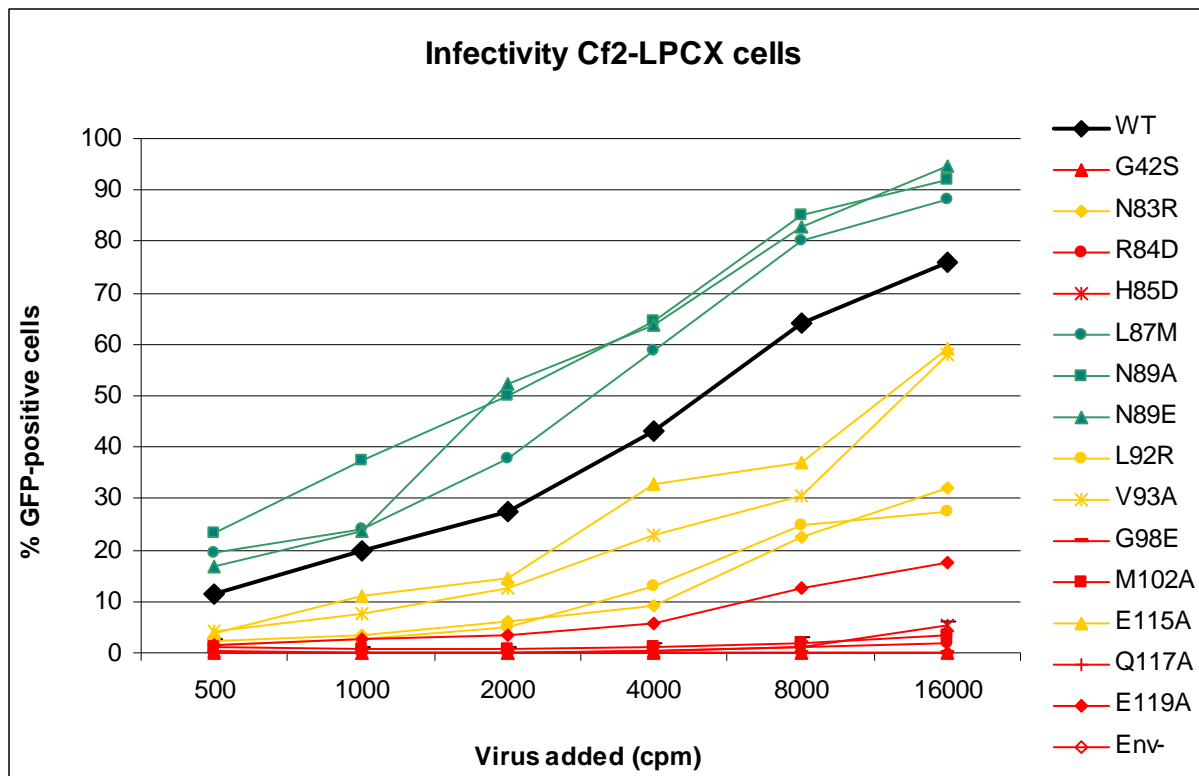
**Figure 2-1. Effects of EIAV CA changes on Gag processing.**

The aligned amino acid sequences of the proline-rich loops of HIV-1 (blue) and EIAV (black) are shown (A). Numbering corresponds to the amino acid number in the HIV-1 CA protein. Red arrows indicate mutated amino acids in EIAV CA, with the corresponding change below the arrow. (B) Cell lysates from 293T cells transfected with plasmids encoding wt and mutant EIAV Gag polyproteins, with positions of weight markers indicated. Total cellular proteins were subjected to Western blotting using an anti-EIAV polyclonal serum. (C) Virion-containing cell supernatants were centrifuged. Solubilized virion pellets were analyzed by Western blotting using an anti-EIAV polyclonal serum.

### Effects of CA changes on EIAV infectivity

To assay the effects of the CA changes on infectivity, Cf2-LPCX cells were challenged with various amounts of wt and mutant EIAV-GFP (Fig. 2-2). Several mutants exhibited levels of infectivity substantially lower than that of wt EIAV (G42S, R84D, H85D, G98E, M102A, Q117A and E119A). Of these mutants, G42S, H85D, G98E, and Q117A show greatly reduced expression or processing of Gag polyproteins, which likely reflects conformational problems that

contribute to their infectivity defects. The three remaining mutants, R84D, M102A and E119A exhibit normal Gag precursor processing and virion production/release. In addition, three mutants (L87M, N89A and N89E) appear to be slightly more infectious than wt EIAV.



**Figure 2-2. Infectivity of EIAV CA mutants in Cf2-LPCX cells.**

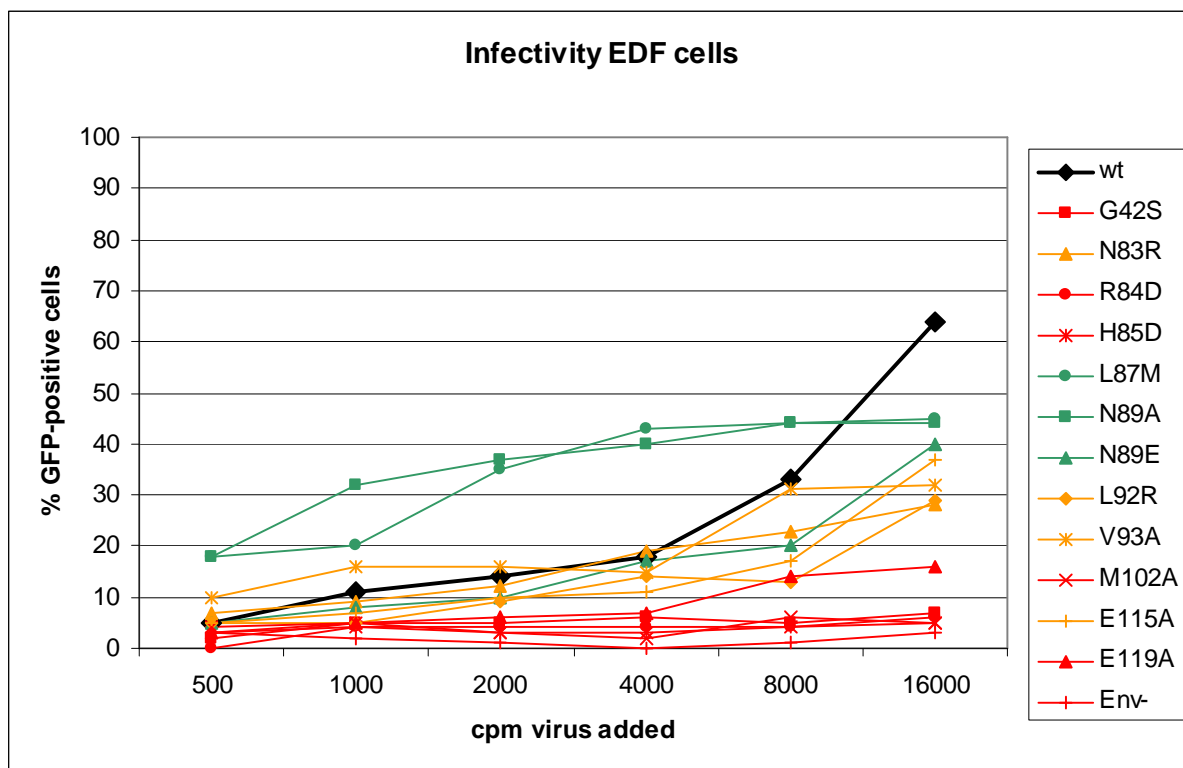
Cf2 cells containing the empty retroviral vector LPCX were challenged with the indicated amounts of wt or mutant EIAV-GFP viruses. After 48 h, the percentage of GFP-positive cells was determined by FACS. Mutants are color-coded based on their percentage of infectivity as compared to wt EIAV (black line). Red: 0-15% infectivity; yellow: 30-75% infectivity; green: higher infectivity than wt EIAV. cpm, counts per minute as determined by reverse transcriptase activity.

As horses are the natural hosts of EIAV, we wished to determine the effects of these CA changes on infectivity in an equine cell line. Equine dermal (ED) fibroblasts were challenged with various amounts of wt and mutant EIAV-GFP. The infectivity profiles of the mutants in the ED cells were very similar to those in Cf2-LPCX cells. Namely, poor expressors/producers were not infectious, while R84D, M102A and E119A exhibited decreased infectivity. L87M and N89A

were slightly more infectious than wild-type EIAV (Fig. 2-3A). However, the N89E mutant, which replicated slightly more efficiently than wt EIAV in Cf2-LPCX cells, did not exhibit this phenotype in ED fibroblasts.

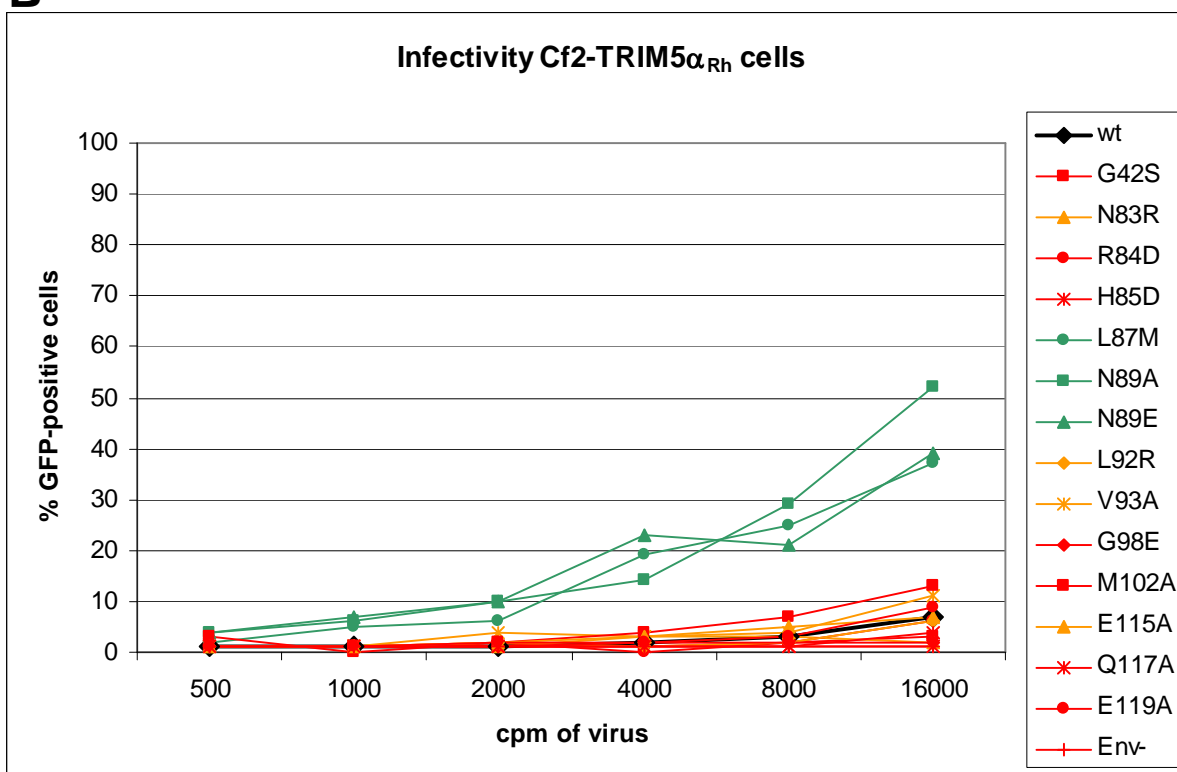
The TRIM5 $\alpha$  protein from rhesus macaques has been shown to restrict EIAV infectivity (71). Cf2 cells expressing the rhesus TRIM5 $\alpha$  protein were challenged with various amounts of wt and mutant EIAV-GFP virus. Most mutants were strongly restricted by rhesus TRIM5 $\alpha$ , similar to wt EIAV. However, the three mutants that exhibited increased infectivity compared to wt EIAV in Cf2-LPCX cells also exhibited less restriction by rhesus TRIM5 $\alpha$  in the matched Cf2 cells (Fig. 2-3B).

**A**



**Figure 2-3. Infectivity of EIAV CA mutants in ED cells and Cf2 cells expressing rhesus TRIM5 $\alpha$ .**



**B**

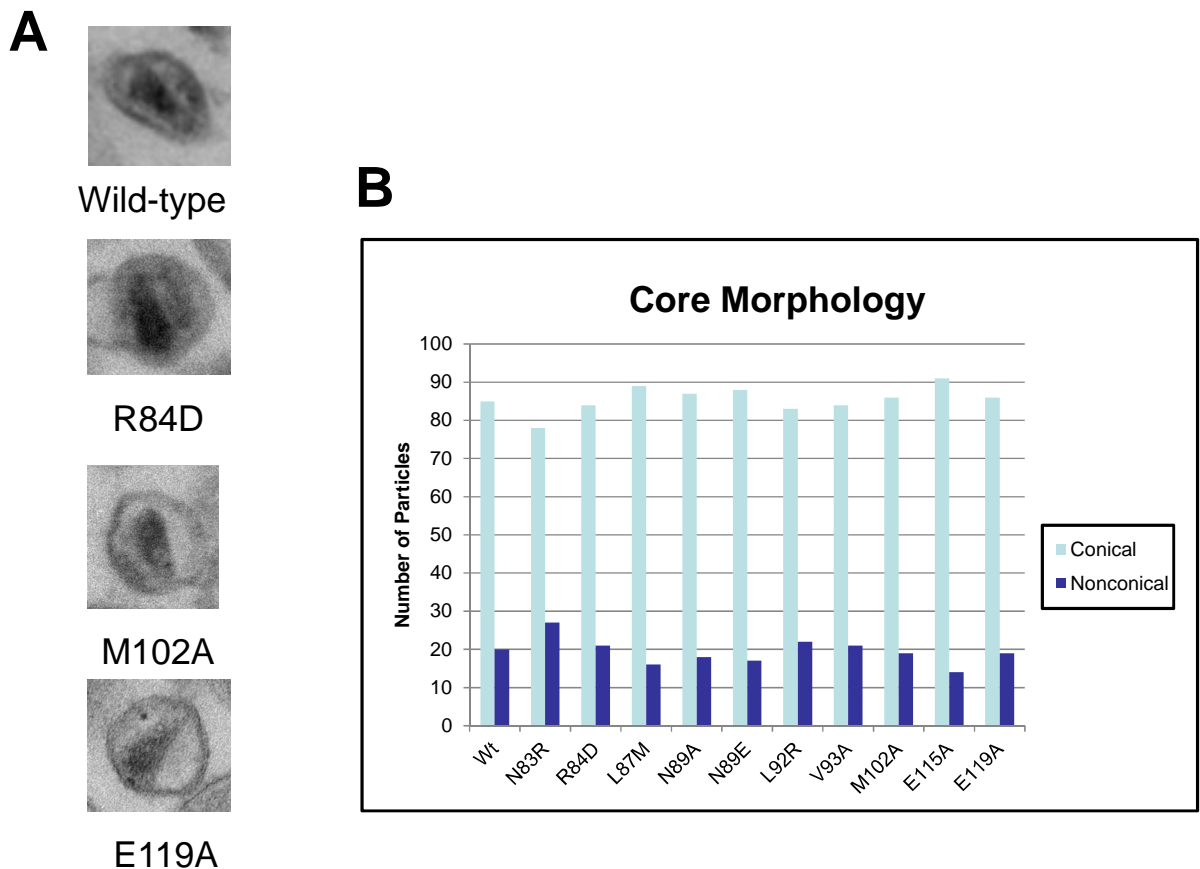
**Figure 2-3 (Continued). Infectivity of EIAV CA mutants in ED cells and Cf2 cells expressing rhesus TRIM5 $\alpha$ .**

Different amounts of wt or mutant EIAV-GFP were used to infect either ED cells (A) or Cf2-TRIM5 $\alpha_{Rh}$  cells (B). The results of a single experiment typical of those obtained in three independent experiments are shown. The color of each CA mutant represents its infectivity in Cf2-LPCX cells (Fig. 2-2). cpm, counts per minute as determined by reverse transcriptase activity.

### Effects of CA changes on EIAV capsid morphology

Some changes in the HIV-1 CA protein have been shown to alter the characteristic conical shape of the mature capsid. These changes have often led to decreases in infectivity (21, 64). To examine whether the changes introduced into EIAV CA affect core formation, we analyzed the morphology of wt and mutant virions by thin-section electron microscopy (EM). All mutants were examined, with the exception of the poor producers G42S, H85D, G98E, and Q117A. Mature, conical cores were observed for the wt and CA mutant viruses (Fig. 2-4A). The ratio of the amount of classical conical cores to cores with nonconical morphology was similar for wt

and mutant virions (Fig. 2-4B). Apparently, the formation and morphology of the mature virion-associated core is not significantly affected by the introduced changes in the CA protein.



**Figure 2-4. Thin-section electron microscopy of EIAV virions.**

Virions were purified from the supernatant of 293T cells transfected with plasmids encoding wt or mutant EIAV Gag/Pol proteins and fixed with glutaraldehyde for thin-section electron microscopy. **(A)** Examples of virions with evident conical cores are shown. **(B)** One hundred five particles were counted and scored as conical or nonconical for each CA mutant. Light blue bars indicate virion particles scored as conical; dark blue bars represent virions scored as nonconical.

### Effects of CA changes on EIAV uncoating in Cf2-LPCX cells

To gain insight into the infectivity defects caused by the CA changes, we studied the uncoating of the wt and CA mutant viruses using a previously developed fate-of-capsid assay (60). This assay allows us to measure the amounts of particulate and soluble forms of retroviral CA present in the cytosol of infected cells. The capsids in the particulate, cytosolic fraction are associated

with viral RNA and also exhibit densities that are comparable to those of intact capsids prepared by detergent treatment of virions (13, 28, 53, 60, 65). The appearance of particulate CA in the cytosol depends upon viral entry and is also affected by the restriction factors TRIM5 $\alpha$  or TRIMCyp (6, 7, 24, 53, 60). VSV-G-pseudotyped virions continue to enter cells up to 16 hours post-infection; therefore the steady state levels of cytosolic CA in the particulate fraction reflect both entry and decay of the capsids. Virus entry is apparently not influenced by the particular CA variant; thus, comparing the steady-state levels of cytosolic CA in the particulate fraction allows an assessment of the conversion of particulate CA to soluble CA, which is measured in the supernatant (SN) fraction. The fate-of-capsid assay also allows a study of the decay of these two forms of CA (6, 60). Because previous studies employing this assay have not used EIAV, we wished to first ascertain the optimal time at which to view the conversion of particulate CA to cytosolic CA. Namely, this point would occur when the signal intensity in the particulate fraction peaks, before it begins to decay. To determine at what time post-infection this point occurs, Cf2-LPCX cells were infected with either VSV-G-pseudotyped EIAV virus, or with EIAV virion-like particles that did not have an envelope glycoprotein (Env-). Infection was allowed to proceed for a given amount of time, after which the cells were harvested and lysed as previously described (60). Figure 2-5 shows the results of this experiment, which creates a time course in which the entry of CA and the conversion of particulate CA (Pellet) to soluble CA (SN) can be followed (Fig. 2-5A). In addition, the band intensity is plotted versus time to show the rise and decay of signal in the particulate fraction and the appearance of CA in the soluble fraction (Fig. 2-5B). Half-lives were calculated for the decay of signal in the pellet fraction (11.3 h post-infection) and the increase in signal in the supernatant fraction (9.7 h post-infection). Based on this experiment, we conclude that 16-20 hours post-infection is the optimal time at

which to harvest the cells, as this is the time period during which the maximum signal of particulate CA is reached.

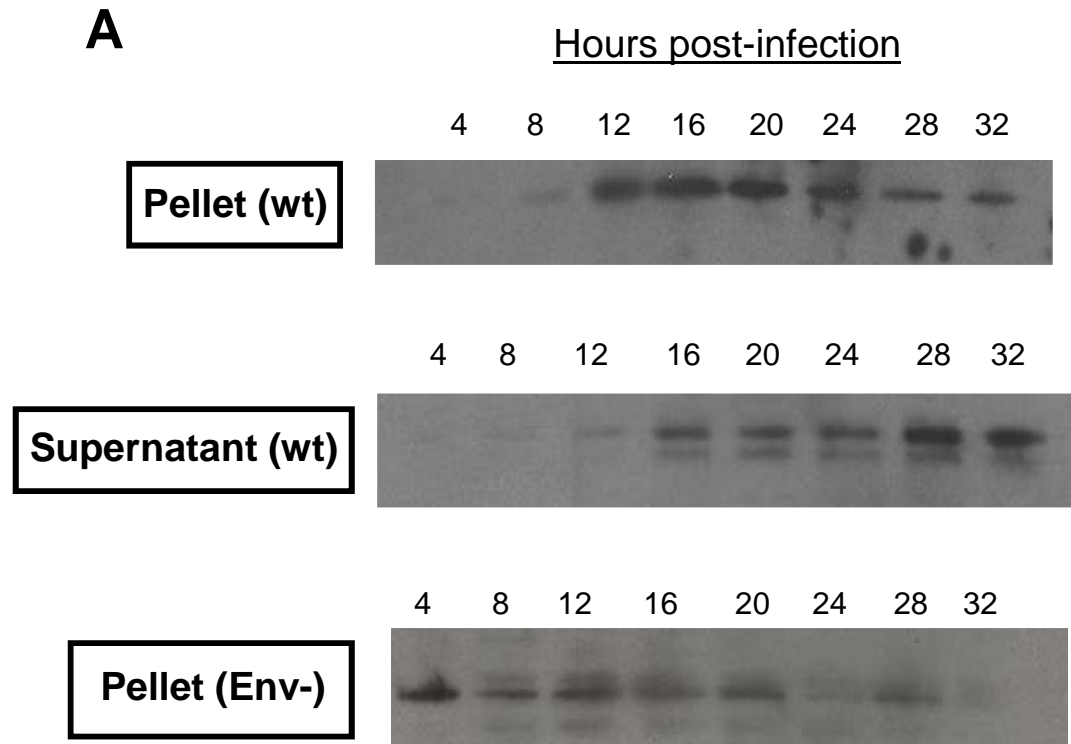
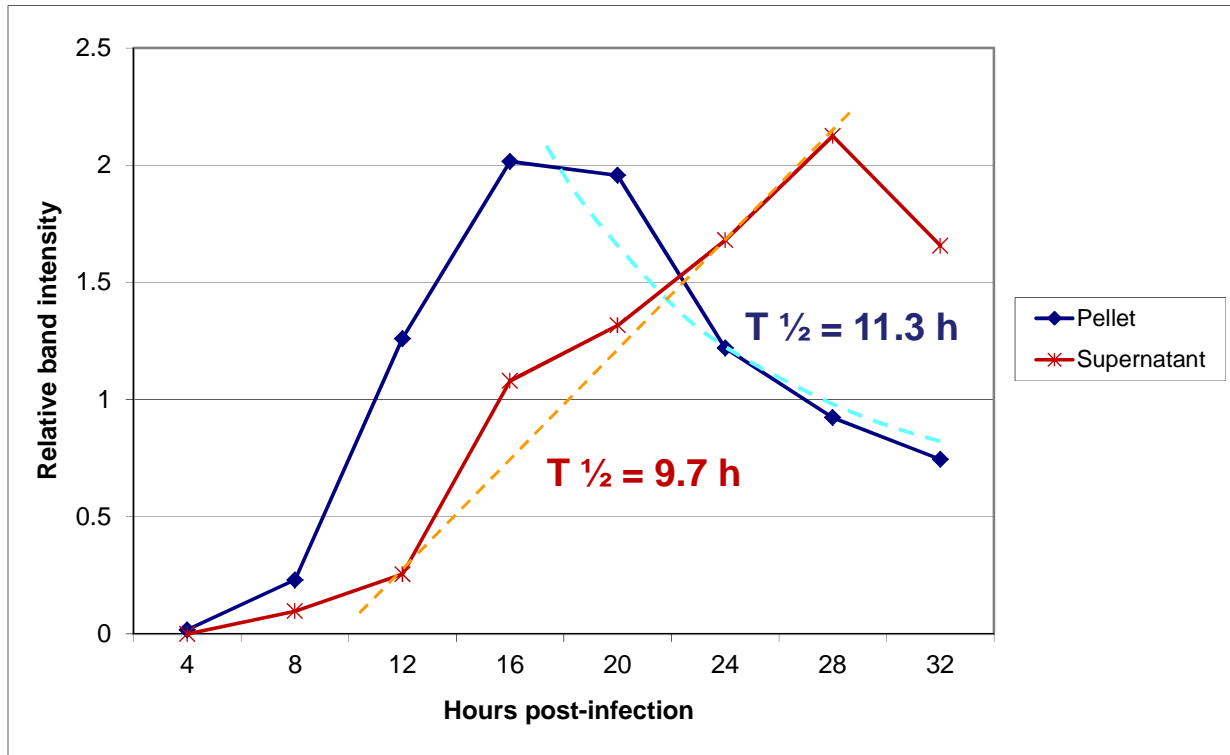


Figure 2-5. A time-course fate-of-capsid experiment in Cf2-LPCX cells infected with EIAV.

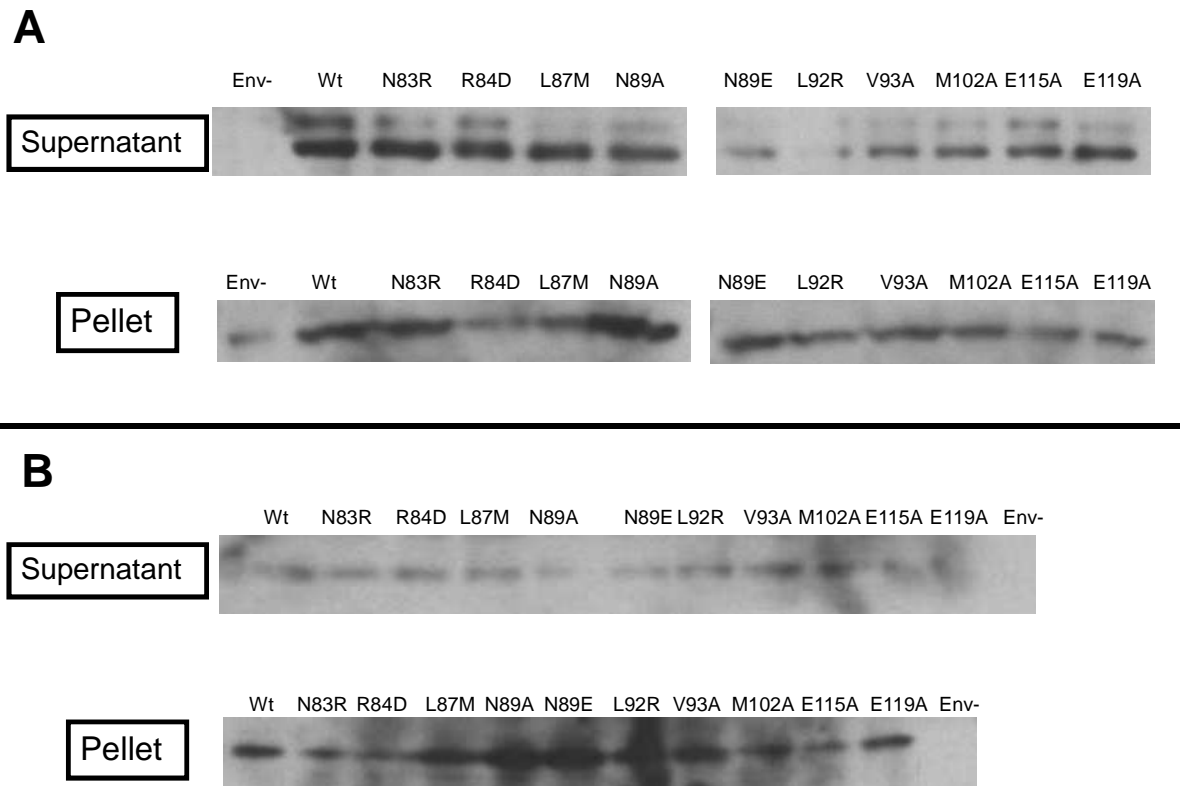
**B**

**Figure 2-5 (Continued). A time-course fate-of-capsid experiment in Cf2-LPCX cells infected with EIAV.**

VSV-G-pseudotyped wt EIAV virions or virions lacking envelope glycoproteins (Env-) were incubated at 37°C with Cf2-LPCX cells for 4 h, at which point the cells were washed and the infection was allowed to proceed for variable lengths of time. Following lysis of the target cells, the cytoplasmic lysates were separated over a 50% sucrose cushion into supernatant (SN) and particulate (Pellet) fractions. (A) The fractions were analyzed by Western blotting, using polyclonal anti-EIAV serum. The particulate fraction of the Env- virions was analyzed and blotted separately from that of the VSV-G-pseudotyped virions. A single film was used for both Pellet and SN fractions of the wt virions and was exposed for 15-30 seconds. The film used for the Env- virions was exposed for 2 minutes in order to see the band signal. (B) The intensity of each band in the Pellet and SN fractions was estimated using band intensity measurements derived from exposed films. The relative intensity of each band was plotted in Excel to give curves showing the increase/decay of signal in the Pellet fraction (blue line) or the SN fraction (maroon line). An exponential curve was fit to time points 16-32 h post-infection for the pellet fraction (cyan dashed line) and the half-life calculated from the equation of the curve. A line was fit to time points 12-28 h post-infection for the supernatant fraction (orange dashed line) and the half-life calculated from the line equation.

To investigate effects of the CA mutations on uncoating, Cf2-LPCX cells were infected for 16 hours with either wt EIAV or the various mutants. Infection was allowed to proceed for 4 h, followed by a wash, and then continued to 16 h, at which time cells were lysed and treated as described above. Figure 2-6 shows two representative experiments. Very little cytosolic p26 CA

protein was observed in the fractions from cells incubated with the non-enveloped control virus (Env-). Any slight differences in the amount of particulate CA in the pellet fraction of wt EIAV versus the CA mutants were not repeatable between experiments. We conclude, therefore, that the introduction of these CA changes does not affect the recovery of particulate CA and that defects in uncoating do not explain the severely abrogated infectivity of some of the CA mutants.



**Figure 2-6. Fate of the capsid in Cf2-LPCX cells infected with EIAV CA mutants.**

VSV-G-pseudotyped wt and mutant EIAV virions were incubated with Cf2-LPCX cells at 37°C for 4 h, washed, and then incubated with fresh media until 16 h post-infection. As a control, Env- virions were included. The separation of cell lysates into particulate (Pellet) and soluble (SN) fractions, and the blotting of each fraction, were done as described for Fig. 2-5. PVDF membranes containing either the Pellet fraction or the SN fraction were exposed on the same piece of film, for 15-30 seconds. (A) and (B) are two independent experiments performed in the same way.

### Effects of CA changes on EIAV reverse transcription

To determine whether the CA mutations affected viral cDNA synthesis, we used a TaqMan-based real-time PCR assay with specific primer and probe combinations, described previously

(25) and detailed in Materials and Methods. Early and late reverse transcripts were analyzed after infection of Cf2-LPCX cells with wt EIAV, the EIAV CA mutants, or Env- EIAV. In a control infection, we challenged cells treated with 150  $\mu$ M AZT with wt EIAV. Cf2-LPCX cells were infected with equal amounts of virus stocks normalized by RT activity. At specific times after infection, cells were harvested by trypsinization and total cellular DNA was isolated. The presence of early DNA products (minus-strand strong-stop DNA) was analyzed by the primer/probe set detailed in Materials and Methods. The results revealed that all mutants produced levels of early RT products similar to those observed for wt EIAV (Fig. 2-7A). For all viruses, the levels of strong-stop DNA increased until about 18 hours post-infection and then began to decrease, suggesting that the EIAV CA mutants do not have any defects in early reverse transcription.

We also examined in parallel the levels of late RT DNA production based on amplification of the *eGFP* gene encoded in the pSIN6.1CeGFPW vector, as described in Materials and Methods. Genomic DNA was isolated from harvested cells as early as 15 minutes post-infection and as late as 36 hours post-infection. Once again, the EIAV CA mutants produced levels of late RT products similar to those observed for wt EIAV (Fig. 2-7B). Taken together, these data demonstrate that the introduction of the CA mutations does not affect viral cDNA synthesis.

**A**

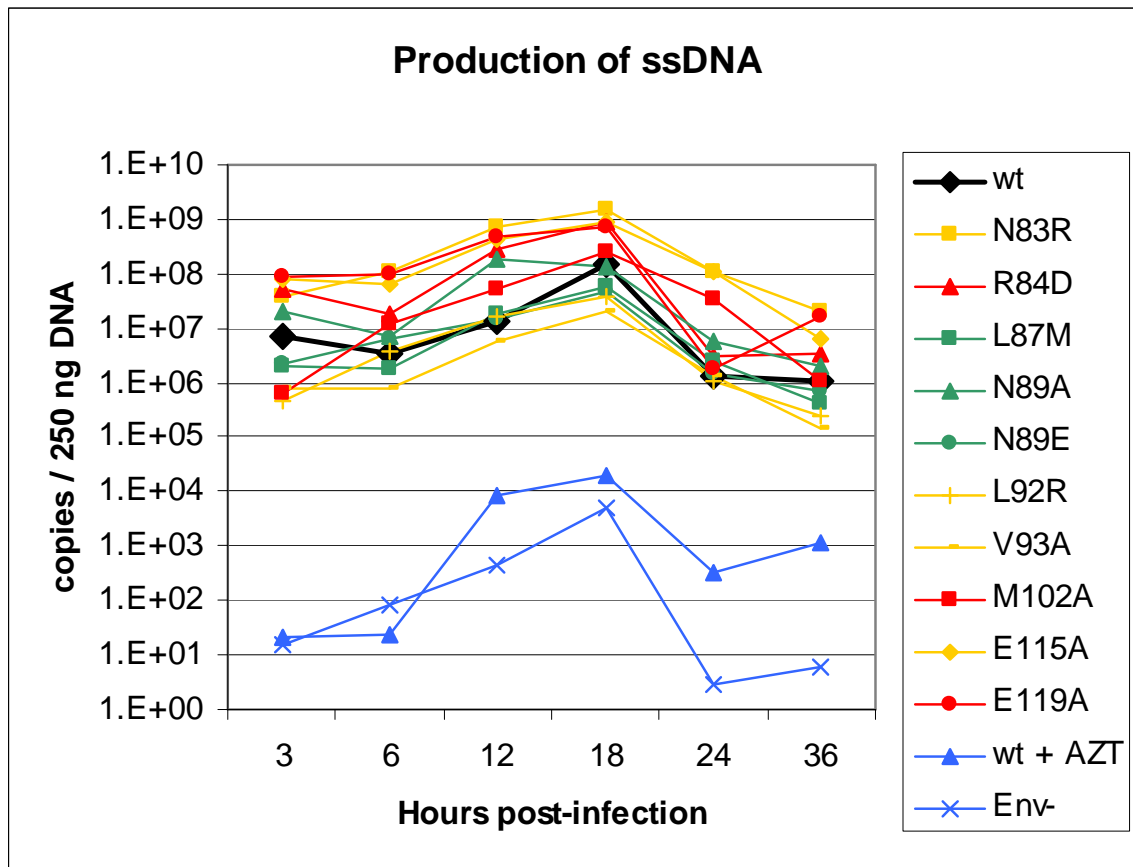
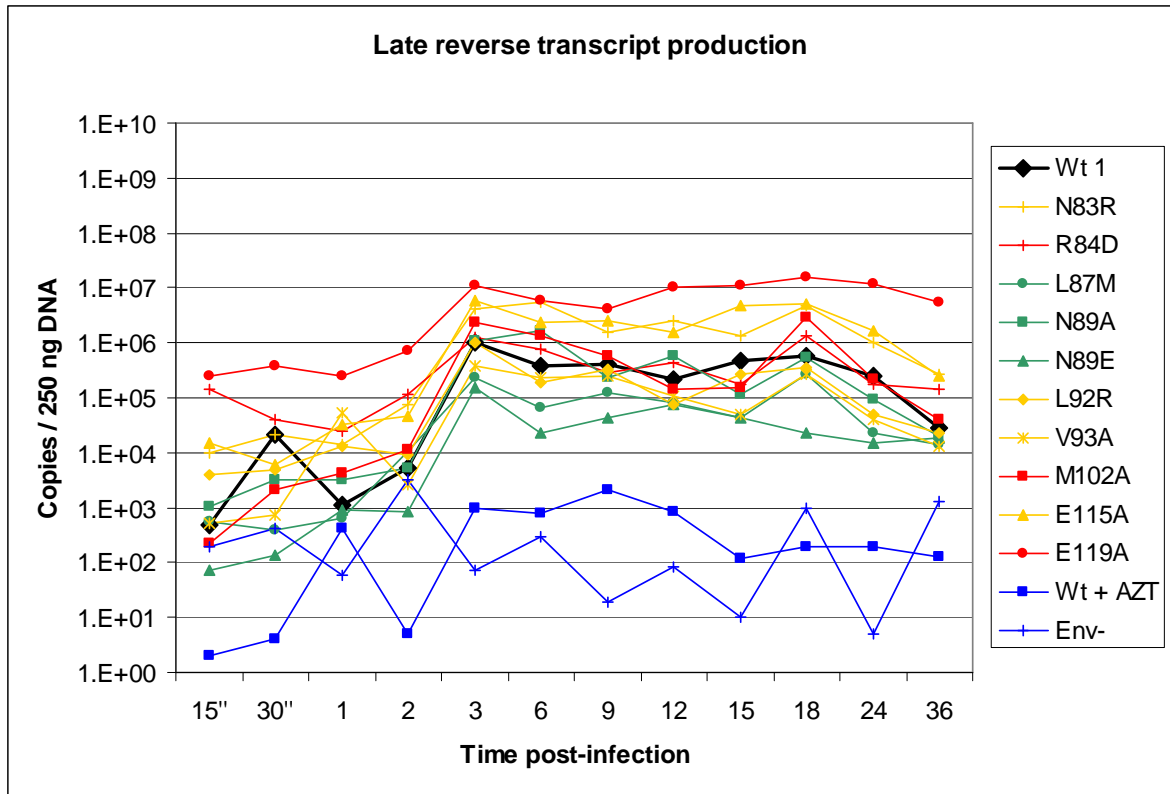


Figure 2-7. Levels of early and late reverse transcripts in infected Cf2-LPCX cells.



**B**



**Figure 2-7 (Continued). Levels of early and late reverse transcripts in infected Cf2-LPCX cells.** VSV-G-pseudotyped EIAV wt or mutant viruses were normalized by reverse transcriptase activity and incubated with Cf2-LPCX cells. In addition, Env- EIAV was included as a negative control. A second negative control consisted of wt EIAV infection of cells treated with 150  $\mu$ M AZT. At the indicated times post-infection, cellular DNA was isolated and quantified for each specific DNA species. Early reverse transcription products (strong-stop DNA; Fig. 2-7A) and late reverse transcription products (*eGFP*; Fig. 2-7B) were quantified using a Taqman-based real-time PCR assay. Data represent the results of a single representative experiment. The color of each CA mutant represents its infectivity in Cf2-LPCX cells (Fig. 2-2). Blue lines correspond to negative controls.

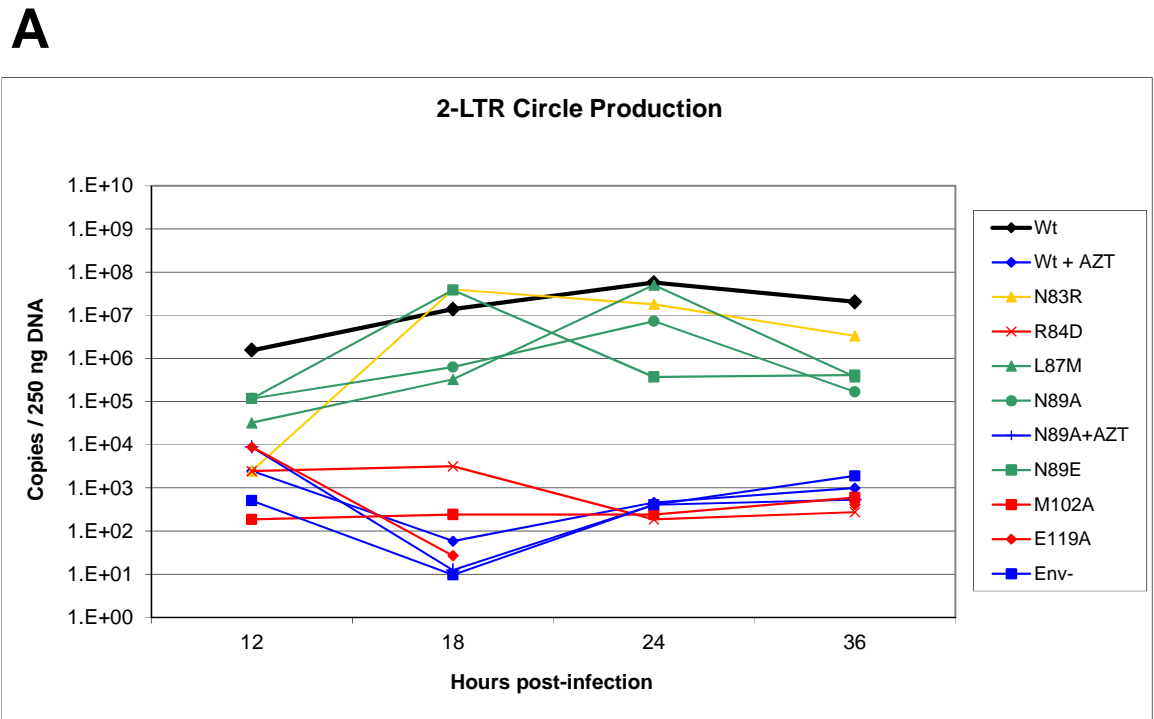
### Characterization of nuclear viral DNA.

Translocation of retroviral DNA from the cytoplasm to the nucleus allows the formation of 2-LTR circles within the nucleus of the infected cell, and thus the amount of 2-LTR circles reflects the efficiency of this translocation (2, 10, 52, 57, 66). To determine whether the EIAV CA changes affected this stage of the viral lifecycle, we measured 2-LTR circular DNA species using a SYBR Green-based quantitative real-time PCR assay with the primer set detailed in

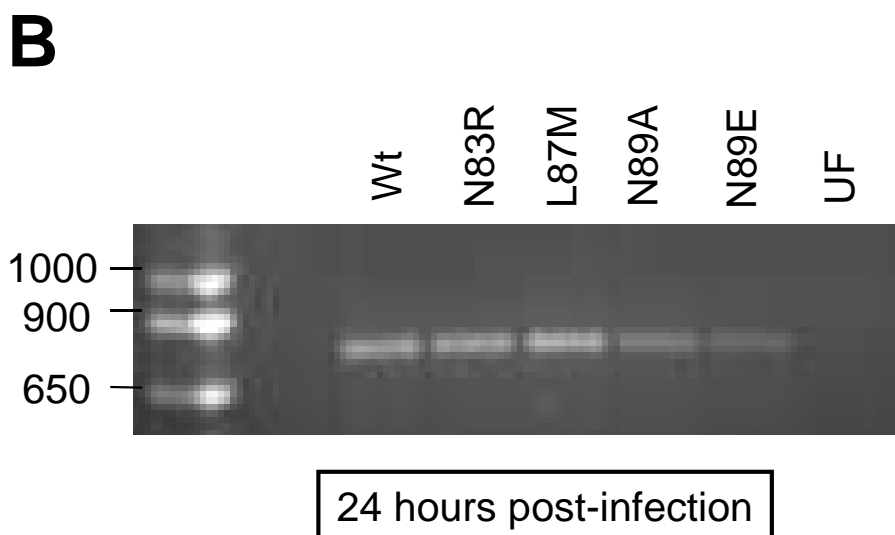
Materials and Methods. These primers span the junction of the 5'LTR and 3'LTR that is created during 2-LTR circle formation, resulting in an amplicon that is approximately 860 nucleotides long. The results of this assay are summarized in Figure 2-8A. Genomic DNA from wt EIAV and EIAV CA mutants representing three different phenotypes (wt infectivity, infectivity slightly higher than wt, and severely abrogated infectivity) was assayed for 2-LTR circle production at 12, 18, 24 and 36 hours post-infection. In addition, the negative controls of wild-type EIAV with AZT-treated cells, EIAV mutant N89A with AZT-treated cells, and Env- wt EIAV were included. EIAV mutants N83R, L87M, N89A and N89E exhibited 2-LTR circle production at levels similar to those of wt EIAV. These four mutants also exhibited infectivity at levels close to or slightly higher than wt EIAV. Conversely, EIAV mutants R84D, M102A and E119A exhibited a significant decrease in 2-LTR circle production as compared to wt EIAV. The amounts of 2-LTR circles produced by these three mutants were similar to the amounts produced in the AZT-treated cells and Env- virus controls, which likely represent background in the PCR assay. Therefore, it appears that mutants R84D, M10A and E119A are severely compromised in 2-LTR circle production. This may indicate that the loss of infectivity observed with these mutants is due to a defect in the ability of these mutants to translocate retroviral DNA from the cytoplasm to the nucleus.

The first step of integration is 3' processing, in which integrase mediates the hydrolysis of GT dinucleotides to create reactive, recessed CA<sub>OH</sub>-3' ends (9). This 3'-processing makes the viral DNA vulnerable to autointegration. Autointegration occurs when these reactive ends attack sites within the viral DNA, resulting in nonproductive deletion or inversion circles (16, 31, 34, 38, 58). Autointegration products are heterogeneous in size, and when amplified by PCR and run on an agarose gel will result in DNA smear (32). In order to verify that autointegration was

not occurring with the EIAV CA mutants, the DNA from the PCR reactions used to amplify the 2-LTR junction was run on an agarose gel (Fig. 2-8B). For all mutants tested, a distinct band of the correct size (860 nt) was observed, indicating that there was no autointegration.



**Figure 2-8. Levels of 2-LTR circles in Cf2-LPCX cells.**



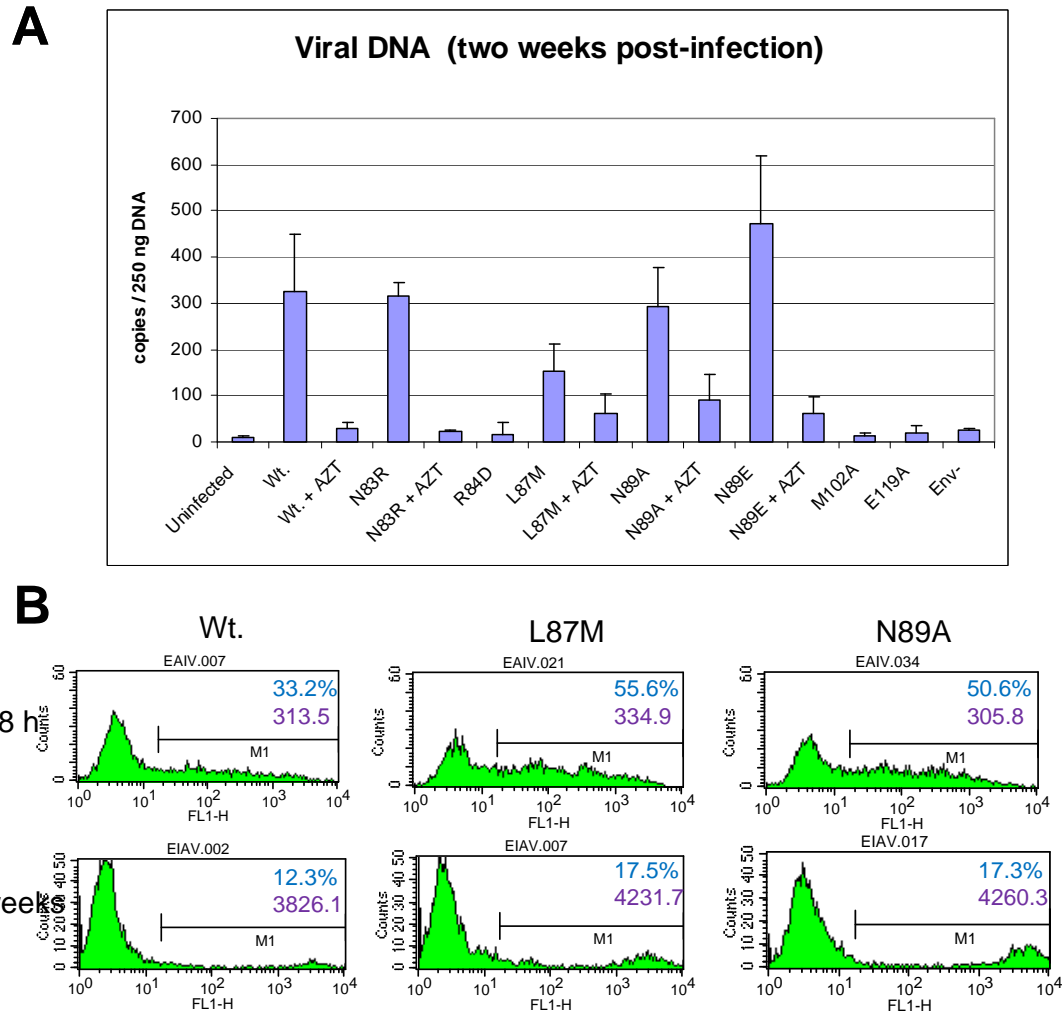
**Figure 2-8 (Continued). Levels of 2-LTR circles in Cf2-LPCX cells.**

(A) VSV-G-pseudotyped wt EIAV or mutant viruses were normalized by reverse transcriptase activity and incubated with Cf2-LPCX cells. In addition, Env- EIAV and wt EIAV infection of AZT-treated cells (150  $\mu$ M AZT) were included as negative controls. At the indicated times, genomic DNA was isolated and quantified for EIAV 2-LTR circles using a quantitative SYBR Green-based real-time PCR assay. (B) The 860 nt fragment created during the PCR for 2-LTR circles. DNA molecular weight standards are marked in the leftmost lane.

### Persistent EIAV viral DNA in Cf2-LPCX cells infected with EIAV CA mutants

To complete the comparative analysis of viral DNA production by the EIAV CA mutants, we next evaluated the presence of persistent viral DNA in Cf2-LPCX cells at two weeks post-infection. Monitoring the presence of viral DNA at this lengthy time period post-infection allows inference of the amount of integrated provirus, as transient DNA forms that are not integrated (e.g. 2-LTR circles) should be lost over time. These data are summarized in Figure 2-9. As expected, those mutants which exhibited very low 2-LTR circle production (R84D, M102A, and E119A) also showed very low levels of persistent viral DNA, comparable to amounts of viral DNA in the negative controls (infection with nonenveloped virus or after AZT treatment) (Fig. 2-9A). These results are consistent with those obtained in the 2-LTR circle assay, suggesting that the R84D, M102A and E119A CA mutants are inefficiently imported into the nucleus of infected cells. All other CA mutants showed levels of persistent viral DNA

similar to wt EIAV virus. Interestingly, the three mutants that were more infectious than wt EIAV (L87M, N89A and N89E) did not consistently demonstrate an increased amount of persistent vDNA. To determine that the increase in infectivity seen with these mutants 48 h post-infection was not due to GFP expression from a transient DNA species lost over the following two weeks, cells from these mutants were subjected to FACS at the 2 week post-infection point before genomic DNA was extracted. Figure 2-9B shows an example of the FACS data. The relative increase in GFP-positive cells for mutants L87M and N89A at 2 weeks post-infection was similar to that seen at 48 h post-infection. As these mutants behaved similarly to wt EIAV in all other infection steps surveyed, our assays may not be sufficiently sensitive to detect the basis for the observed increase in infectivity.



**Figure 2-9. The presence of persistent viral DNA in Cf2-LPCX cells 2 weeks post-infection.**

VSV-G-pseudotyped EIAV wild-type or mutant viruses were normalized by RT activity and incubated with Cf2-LPCX cells. Env- EIAV was included as a negative control. As an additional control, 150  $\mu$ M AZT was added to cells incubated with some of the viruses. Infection was allowed to proceed for 2 weeks. Cells were continuously split throughout this time period as they reached confluency. (A) At the end of 2 weeks, genomic DNA was isolated. The presence of persistent viral DNA was assayed using a Taqman-based quantitative real-time PCR assay and the primer/probe set described in Fig. 2-7B, which amplifies a fragment from the *eGFP* gene. (B) FACS profiles of wt. EIAV and mutants L87M and N89A. The top row contains profiles from cells 48 h after infection. The profiles in the bottom row are from cells 2 weeks post-infection, at which time genomic DNA was harvested and real-time PCR used to detect the presence of viral DNA (see A above). Blue numbers within each window indicate the percentage of GFP-positive cells. Purple numbers indicate the mean fluorescence intensity values.

## **Discussion**

In this study, we have created a panel of EIAV CA mutants and attempted to ascertain what effects the introduced changes have on the early stages of EIAV infectivity. We have determined that the introduction of single amino-acid residue changes in and around EIAV's proline-rich loop can dramatically alter infectivity. Three mutants in this study exhibited a severe decrease in viral infectivity that appears to occur during the step of nuclear import of the pre-integration complex. Three other mutants exhibited infectivity that was slightly better than that of wt EIAV, although no significant differences from the wt virus were detected in the early post-entry steps of infection (summarized in Table 2-1).

Mutant	CL	VLPs	EM	Cf2-LPCX	Cf2-TRIM5 $\alpha_{Rh}$	Fate of CA	Early [x10 <sup>7</sup> ] (12 h)	Late [x10 <sup>5</sup> ] (12 h)	2-LTR (24 h)	vDNA (2 wk)
Wt	++	++	+	++	-	+	1.3	2.2	5.7 x 10 <sup>7</sup>	324
G42S	+	+		-	-					
N83R	++	++	+	+	-	+	75.7	25.9	1.7 x 10 <sup>7</sup>	315
R84D	++	++	+	-	-	+	28.5	4.3	186	17
H85D	+	+		-	-					
L87M	+	+	+	+++	++	+	1.8	0.83	5 x 10 <sup>7</sup>	154
N89A	++	++	+	+++	++	+	1.8	5.9	7.3 x 10 <sup>6</sup>	293
N89E	++	++	+	+++	++	+	1.4	0.76	3.7 x 10 <sup>5</sup>	473
L92R	+	++	+	+	-	+	1.6	0.76		
V93A	+	+	+	+	-	+	0.59	1.1		
G98E	-	-		-	-					
M102A	++	++	+	-	-	+	5.3	1.4	241	14
E115A	++	+	+	+	-	+	44.4	15.7		
Q117A	-	-		-	-					
E119A	+	++	+	-	-	+	48.8	99.5	1174	21

**Table 2-1. A summary of the effects of the EIAV CA changes.** CL: expression/processing in cell lysates. VLPs: production of virus-like particles. EM: production of conical cores viewed using thin-section electron microscopy. Cf2-LPCX, Cf2-TRIM5 $\alpha_{Rh}$ : infectivity in these cell types measured by GFP positivity. Fate of CA: uncoating as measured in the fate-of-capsid assay. Early: production of early reverse transcripts measured by qPCR 12 h post-infection. Late: production of late reverse transcripts measured by qPCR 12 h post-infection. 2-LTR: production of 2-LTR circles measured by qPCR 24 h post-infection. vDNA: persistent proviral DNA measured by qPCR 2 weeks post-infection.

When the EIAV CA mutants were used to infect either canine-derived Cf2-LPCX cells or equine ED cells, we observed phenotypes that we classified into three groups: mutants that were defective (0-15% relative infectivity at highest multiplicity of infection), mutants exhibited infectivity levels 30-75% of that of wt EIAV, and those that were slightly more infectious than



wt virus. The severe infectivity defects of several mutants could be explained either by poor Gag polyprotein expression (H85D, G98E, Q117A) or poor Gag precursor processing (G42S). These mutants may have altered conformations due to the changes in the proline-rich CA loop. All of the remaining mutants surveyed by thin-section electron microscopy exhibited typical cone-shaped core morphology. The incidence of cores with aberrant shapes was similar for these mutants and wt EIAV, indicating that introduction of most of the changes in the CA proline-rich loop does not affect viral core morphology.

Application of the fate-of-capsid assay allowed measurement of levels of particulate CA in cells infected with CA mutants, providing a window into the uncoating process. A fate-of-capsid time course experiment determined the optimal time at which to harvest the cells post-infection and also provided information about the rate of conversion of particulate CA to soluble CA, i.e. the rate of EIAV uncoating. Under the conditions of our assay, by 20 h post-infection, viral entry has diminished. This allows measurement of the decay constant for EIAV uncoating. The implication of this result is that, even at 36 h post-infection, long after nuclear import of pre-integration complexes, a small percentage of capsids have not yet uncoated. Whether these capsids or partially uncoated capsids are transported into the nucleus is unclear, but the persistent presence of the CA helps to explain its role in nuclear import.

Three EIAV CA mutants (R84D, M102A and E119A) exhibited severe defects in infectivity despite levels of expression, Gag processing and virion production that were generally comparable to those of wt EIAV. Only E119A exhibited a lower level of expression than wt EIAV in the cells, but released virus efficiently. When these EIAV CA mutants were used to infect cells, we did not observe significant differences between the mutants and wt EIAV, either in the amount of particulate CA present or in the rate of conversion to soluble CA. To look for

possible defects in reverse transcription, we used quantitative real-time PCR to measure levels of both early and late reverse transcripts at various time points after infection. All mutants tested, regardless of their infectivity phenotypes, produced similar levels of early and late reverse transcripts at similar rates. The combined results of fate-of-capsid experiments and real-time PCR suggest that the infectivity defects we observed are not due to problems with uncoating or reverse transcription.

The 2-LTR circular form of DNA is thought to be a reliable indicator of transport of retroviral PICs from the cytoplasm to the nucleus (2, 10, 52, 57, 66). In this study, we observed that the three replication-defective mutants (R84D, M102A and E119A) exhibited lower production of 2-LTR circles. In addition, an experiment to measure the amount of viral DNA present two weeks post-infection (indicative of integrated provirus) showed a severe decrease in the amount of viral DNA present. Taken together, these data suggest a defect in the step of transport of the EIAV PIC to the nucleus.

Previous studies have found HIV-1 CA mutations that appear to complete uncoating and reverse transcription normally but are defective in 2-LTR circle production (13, 25, 44). Because the machinery required for nonhomologous end-joining (required for 2-LTR circle formation) is located in the nucleus (20), a defect that occurs after reverse transcription but prior to nuclear entry of the PIC manifests itself in lower 2-LTR circle production and is associated with lower amounts of integrated provirus. Recently, some HIV-1 CA mutants that are defective for 2-LTR circle production have been shown to be unable to interact with certain host nuclear import factors. For example, the N74D CA mutant affects HIV-1 sensitivity to TNPO3 knockdown, and HIV-1 chimera viruses that contain murine leukemia virus CA protein exhibit differential sensitivity to knockdown of Nup153 (1, 5, 29, 44, 56).

Although we have determined the viral lifecycle step that is defective in these EIAV CA mutants, further studies will be required to determine how these mutations affect nuclear import. Thus far, recent studies have shown that EIAV is sensitive to depletion of both TNPO3 and NUP153. In addition, the cargo-binding domain of TNPO3 is critical for the nuclear import of divergent lentiviruses. However, mutational analysis of CA determinants critical to nuclear import has focused mainly on the HIV-1 CA. Here, we have shown that EIAV CA also appears to be a critical determinant for nuclear import, which supports the previous HIV-1 studies. However, we currently do not have insight as to why these particular CA changes result in this defect for EIAV. Future studies should look at the requirements of host factors critical for HIV-1 nuclear import and seek to apply these requirements to EIAV. In addition, further work can be done from the viral side to determine the mechanism of import defects for these three mutants.

Finally, we have identified three amino acid changes to EIAV CA that cause the mutant virus to replicate slightly better than wt EIAV. The mutants L87M, N89A and N89E do not appear substantially different from wt virus in terms of 2-LTR circle production or levels of integrated provirus. Because we have not identified a step in the infectivity cycle for these three mutants that differs significantly from wt EIAV, it is difficult to account for the increased infectivity they display in Cf2-LPCX cells (Fig. 2-2, 2-3). One possible explanation is that our assays, including the fate-of-capsid assay to measure uncoating and real-time PCR assays to measure production of early and late transcripts, 2-LTR circles and levels of integrated provirus, are not sensitive enough to detect differences between wt EIAV and these three mutants that lead to the observed increase in infectivity. Future experiments on these three mutants should first employ more sensitive real-time PCR assays to determine whether there are in fact differences in production of any of the DNA species assayed above which could account for their increased replication. If in

fact these CA mutants do not differ from wt virus in any of the steps assayed above, there is the unlikely possibility that they preferentially integrate into host genomic sites where they are more actively transcribed. EIAV, like HIV-1, shows a preference for integration into transcription units (22, 43). It may be possible that these three particular CA mutations can in some way affect integration site preference in a manner that leads to enhanced GFP expression.

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# CHAPTER 3

## **DISCUSSION**

The retroviral capsid (CA) protein plays a role in several early steps of the viral life cycle. Several studies have addressed the role of CA during the poorly-defined uncoating step, which occurs shortly after the viral core enters the target cell cytoplasm. Lentiviruses like HIV-1 and EIAV contain a conical core within the lipid membrane of the virion. This core is composed of CA protein hexamers that form a hexagonal structure termed a fullerene cone (5, 10-12, 15, 21). In HIV-1, proper stability of this conical core has been shown to be critical for infection. Forshey *et al.* introduced point mutations into the HIV-1 CA that altered core stability in either a positive or negative direction, as assayed by *in vitro* experiments. Any alteration in stability impaired infectivity at a step prior to or during reverse transcription (7). This suggests that there is an optimal stability to the incoming core that is critical for the early steps of uncoating and/or reverse transcription. In addition, several groups have attempted to purify native core complexes from HIV-1 virions with varying degrees of success (1, 7, 17, 27, 35) . In most cases, these purified complexes were extremely labile and disassembled upon the addition of buffer. These studies suggest that the HIV-1 core complex exists in a state of metastability and that small alterations to this stability can have deleterious effects on infectivity.

The retroviral CA protein has also been shown to be the target of certain cellular factors that affect the uncoating process. TRIM5 $\alpha$  is a well-known example of a restriction factor that negatively modulates uncoating through its interaction with CA and therefore decreases viral infectivity. TRIM5 $\alpha$  targets the incoming capsid and causes an accelerated disassembly of core complexes (26, 30). The deleterious effects of accelerated uncoating and altered capsid stability suggest that proper temporal regulation is critical to productive uncoating.

Cyclophilin A (CypA) is another host cell factor that modulates uncoating through its

interaction with HIV-1 CA. CypA binds the HIV-1 CA N-terminal proline-rich CypA-binding loop (8, 9, 13, 24, 32). This loop is exposed on the surface of the assembled HIV-1 capsid. Residues Gly89 and Pro90 are particularly critical for CypA binding (3, 29, 34, 39). Disruption of CypA-CA binding, whether through changes in amino acid residues or the addition of the CypA-CA binding inhibitor cyclosporine A (CsA), results in an infection block after entry but prior to reverse transcription; therefore the CypA-CA interaction appears to be involved in modulation of uncoating. Li *et al.* have demonstrated that disruption of CypA-CA binding causes changes in the levels of cytosolic, particulate CA. The amount of particulate CA recovered from target cell cytosol is correlated to viral infectivity, with lower amounts of particulate CA being recovered from CA mutants that are poorly infectious. However, disruption of the CypA-CA interaction can cause different phenotypes depending on target cell type (22).

More recent studies have shown that the role of CA does not end at the uncoating step. Lentiviruses are able to infect nondividing cells such as macrophages and quiescent T cells. This suggests that the preintegration complex (PIC) must be actively transported into the nucleus. The key viral determinant for nuclear import was recently mapped to CA (36-38). Recent genome-wide siRNA screens have identified host nuclear import factors that are critical for HIV-1 infectivity, including nuclear pore complex components NUP153, NUP358 and NUP155 and the nuclear import protein transportin 3 (TNPO3) (2, 18, 23, 25, 28, 33, 40). The requirements for TNPO3 and NUP153 have both been shown to map to CA, and knockdown of either impairs lentiviral infection at the nuclear import step.

The CA protein of EIAV is quite similar to that of HIV-1. The two proteins share 30%

sequence identity and significant structural similarities (9, 15). Although it has been difficult to purify HIV-1 cores, EIAV cores may be prepared fairly easily from detergent-stripped virions, suggesting that the EIAV core may be more stable than that of HIV-1 [(19, 27), Appendix]. In addition, EIAV also has a proline-rich loop located between helices 4 and 5 of the N-terminal domain. This loop is presumed to be exposed on the surface of the assembled EIAV CA. Due to the similarities between the two CA proteins and the presumed greater stability of EIAV CA, we hypothesized that EIAV CA would be an attractive candidate with which to study the effects of CA changes on lentiviral infectivity. In particular, we hypothesized that introducing changes into EIAV's proline-rich loop would yield interesting phenotypes and that effects of the CA mutations would manifest themselves at the uncoating step. Similar amino acid changes in that region of HIV-1 CA have provided insights into HIV-1 infectivity and the uncoating process in particular. In this study, we have introduced amino acid changes in and around EIAV's CA helix 4-5 proline-rich loop and then followed the early infection steps of viruses containing these changes. Based on the results of this study, we have concluded that alterations in this region of EIAV CA can dramatically alter viral infectivity; however, infection is blocked at the nuclear import step and not at uncoating.

Several of the amino acid substitution mutations resulted in defects in cellular expression or Gag processing. These defects were usually correlated with extremely low levels of infectivity in Cf2-LPCX cells. However, three mutants that exhibited normal expression and Gag processing also had severely abrogated infectivity (R84D, M102A, E119A). Interestingly, three other amino acid changes resulted in viruses that were more infectious than wild-type (wt) EIAV in Cf2-LPCX cells (L87M, N89A, N89E). All mutants appeared to form morphologically

normal cores at levels similar to wild-type EIAV, suggesting that these amino acid changes did not affect core morphology.

Based on this preliminary data, we found the CA changes created viruses that expressed one of three infectivity phenotypes in Cf2-LPCX cells: poorly infectious, slightly more infectious than wt EIAV, or infectious at a level similar to wt. Since horses are the natural host of EIAV, we also wished to test infectivity in an equine cell line. Overall, a very similar pattern was observed in infection of equine dermal (ED) fibroblasts. Those CA mutants that were defective in Cf2-LPCX cells were also defective ED cells, while those that were similar to wt EIAV in Cf2-LPCX cells were also similar to wt EIAV in ED cells. Of the three mutants that exhibited better infectivity than wt in Cf2-LPCX cells, two of them were also more infectious than wt in ED cells. Finally, we also wished to test the mutants for sensitivity to rhesus TRIM5 $\alpha$ , a potent restrictor of EIAV infection. All CA mutants tested were restricted by rhesus TRIM5 $\alpha$  at levels comparable to the restriction of wt EIAV, with the exception of the three mutants that were more infectious than wt in Cf2-LPCX cells. These three mutants were also less sensitive to the restricting effects of rhesus TRIM5 $\alpha$ .

We had hypothesized that these CA changes might affect viral uncoating, as similar amino acid changes in the HIV-1 CypA-binding loop have been shown have effects on uncoating that are deleterious to infectivity (7, 22). To test this hypothesis, we used the fate-of-capsid assay to monitor the conversion of particulate CA (representative of intact viral cores) to soluble CA (representative of disassembled cores). Overall, we saw no significant deviations from the wt EIAV uncoating pattern for any of these CA mutants. Data examining levels of early and late reverse transcripts further supported this result. Levels of early and late reverse transcripts were



close to that of wt EIAV for all CA mutants tested.

Thus far the three defective mutants and the three mutants with increased infectivity behaved similarly to wt EIAV in the early steps of uncoating and reverse transcription. Due to recent studies implicating CA in nuclear import, we hypothesized the CA changes could affect this step and therefore account for differences in infectivity. For the defective mutants R84D, M102A and E119A, this was observed. These three mutants exhibited decreased production of 2-LTR circles and also showed very low levels of persistent viral DNA (representative of integrated provirus) two weeks post-infection. As the formation of 2-LTR circles requires nuclear DNA repair enzymes, the decreased amount of 2-LTR circles suggests that the PIC is not entering the nucleus, which in turn leads to reduced amounts of integrated proviral DNA.

TNPO3 has recently been identified as a cellular host factor involved in nuclear import of HIV-1. Knockdown of TNPO3 in HIV-1-infected cells reduces the amount of 2-LTR circles. However, knockdown has no effect on the amount of 2-LTR circles in cells infected with murine leukemia virus (MLV) (4, 6). As a gammaretrovirus, MLV requires breakdown of the nuclear envelope during mitosis to access the nucleus and is not actively transported into the nucleus through the nuclear pore channel (NPC) like lentiviruses. TNPO3 is a member of the karyopherin  $\beta$  protein family that imports serine/arginine-rich splicing factors (SR proteins) into the nucleus (16). TNPO3 has three protein domains: an amino-terminal Ran-GTP binding domain, a central nuclear pore channel binding domain, and a carboxy-terminal cargo binding domain (31). Logue *et al.* have shown that the cargo-binding domain is required for HIV-1 nuclear import. In addition, they have demonstrated that several lentiviruses, including SIV<sub>MAC</sub>239 and EIAV, are also dependent on TNPO3 and its cargo-binding domain for nuclear

import (23).

Another factor required for nuclear import of HIV-1 is the NPC component NUP153. Matreyek *et al.* have demonstrated that the requirement for NUP153 also maps to HIV-1 CA. NUP153 knockdown results in decreased 2-LTR circle production. Replacing HIV-1 CA with MLV CA rendered the chimeric virus insensitive to NUP153 depletion. In addition, NUP153 is required for infection with lentiviruses SIV<sub>MAC</sub>, SIV<sub>AGM</sub>Tan, SIV<sub>AGM</sub>Sab, HIV-2<sub>ROD</sub> and EIAV (25).

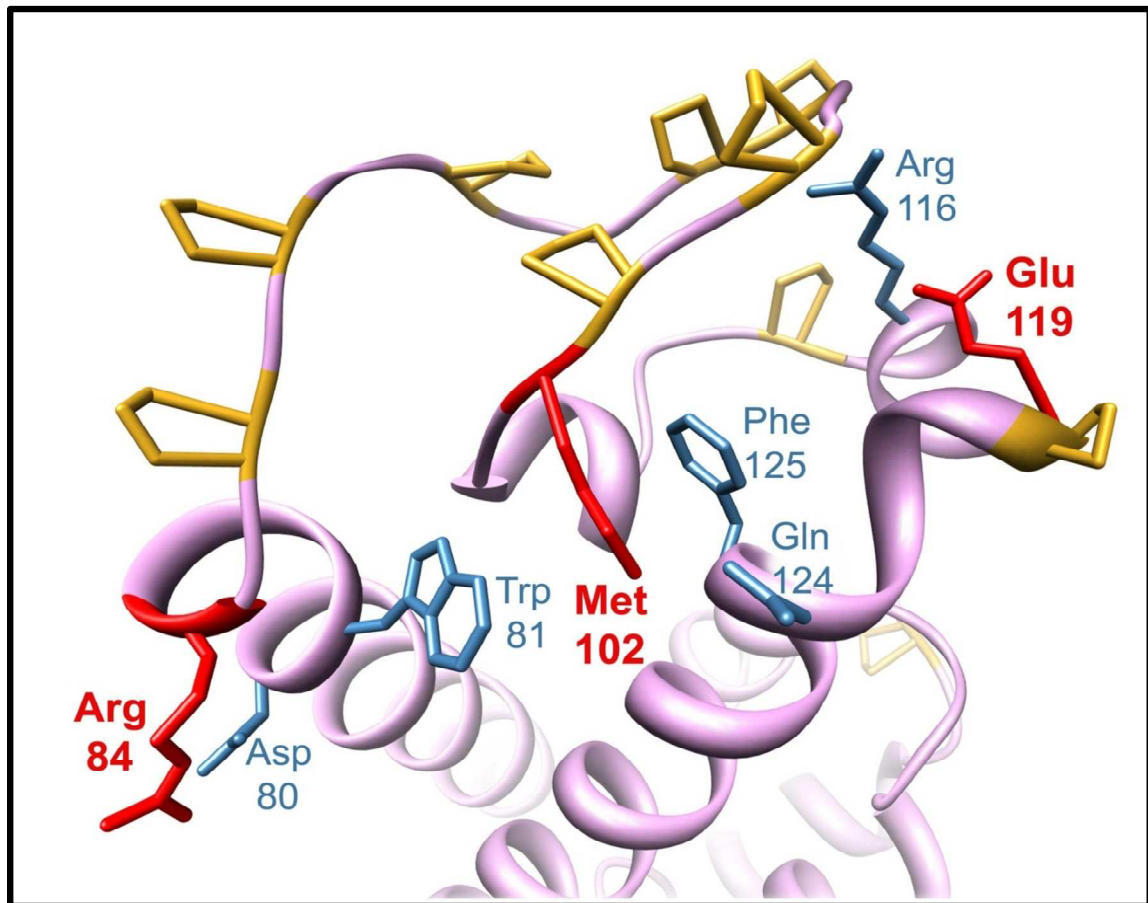
The HIV-1 CA mutant N74D does not require TNPO3 or NUP153 for nuclear import and can infect nondividing cells. The N74D mutant was first identified in a selection for HIV-1 variants that were resistant to the dominant-negative restriction factor mCPSF6-358 (20). mCPSF6-358 is a fragment of CPSF6, a cellular polyadenylation factor. It blocks nuclear import of HIV-1, HIV-2 and SIV. Expression of mCPSF6-358 leads to decreased 2-LTR circle production in infected cells. The HIV-1 CA mutant N74D is resistant to mCPSF6-358. Lee *et al.* have demonstrated that CPSF6-358 binds wt HIV-1 CA-NC complexes more efficiently than it binds HIV-1 N74D CA-NC complexes (20). mCPSF6-358 accumulates in the cytoplasm, and Lee *et al.* speculate that the interaction of mCPSF6-358 with CA could block interactions with critical host nuclear import factors like TNPO3.

Although N74D is insensitive to both TNPO3 and NUP153 knockdown and to the negative effects of mCPSF6-358, interpretation of results obtained using this mutant can be complicated due to HIV-1 CA binding CypA. The N74D mutation increases dependence on CypA, and CsA treatment of cells blocks N74D infection (2). However, adding the

compensatory mutation G89V, which creates a CA that cannot bind CypA (N74D/G89V), renders the double mutant insensitive to CsA. In addition, the N74D mutant cannot infect macrophages due to a block at reverse transcription (2). Finally, HIV-1 CA mutants A92E and G94D, which exhibit a cell-type-specific CypA requirement, became insensitive to NUP153 depletion upon CsA treatment (25). CypA-CA binding and its role in HIV-1 nuclear import appears to be a complicated issue that can be affected by parameters such as CsA treatment, cell type, and amino acid changes.

Thus far, research has shown that EIAV infection, like HIV-1 infection, is abrogated by knockdown of TNPO3 or NUP153, suggesting that EIAV's requirement for nuclear import factors is similar to that of HIV-1. As EIAV does not bind CypA, EIAV sensitivity to nuclear import factor depletion is more straightforward, and requirements for particular proteins are not contingent upon CypA binding. Therefore, EIAV is an attractive virus to use in further studies of the role of CA in nuclear import and host proteins required for it. Current research has focused on EIAV and nuclear import machinery very broadly, only showing that EIAV is sensitive to depletion of TNPO3 and NUP153. An EIAV CA mutation that renders the virus insensitive to knockdown of either of these factors (analogous to HIV-1 N74D) has not yet been identified. However, this study has identified three EIAV CA mutants that are defective in nuclear import. Further studies of these particular mutants should attempt to determine the reason for this defect. An examination of the position of the three mutants within the loop region suggests a possible explanation (Fig. 3-1). Each mutated residue (red) is located near a proline residue (ochre). In addition, the amino acid substitutions introduced create charge changes which may disrupt critical acidic-basic interactions with nearby side chains (red and blue),

thereby changing the conformation of the loop.



**Figure 3-1.** The positions within the proline-rich loop of the three amino acid substitutions that cause nuclear import defects. Red: substituted amino acid residues. Ochre: proline residues. Blue: unchanged amino acid residues whose side chains may interact with those of the substituted residues in wt EIAV.

In addition, ongoing studies examining the requirements of host factors critical for HIV-1 nuclear import should include EIAV. Due to the absence of CypA, EIAV CA may yield clearer data relating to questions about host factor-CA interaction and sensitivity to host factor knockdown. In addition, the natural host cell type of EIAV infection is the quiescent macrophage, and the ability of EIAV CA mutants to infect this cell type should give a clear indication of the effects of CA changes on infection of nondividing cells and therefore

provide some insight into effects on nuclear import.

Unfortunately, we were unable to observe any differences between wt EIAV and the CA mutants L87M, N89A and N89E, all of which were more infectious than wt in Cf2-LPCX cells. These mutants did not exhibit any significant differences in uncoating as measured by the fate-of-capsid assay. They also did not produce significantly different levels of early or late reverse transcripts or 2-LTR circles. Finally, the amount of proviral DNA measured two weeks post-infection was similar to that of wt. Comparing the FACS data of these mutants from 48 h post-infection and 2 weeks post-infection shows that the increased GFP positivity persists, suggesting that the higher fluorescence observed for these mutants in Cf2-LPCX cells is not due to epigenetic GFP expression from some transient DNA species. Thus far, the experiments in this study have not been able to determine why mutants L87M, N89A and N89E appear to be more infectious than wild-type EIAV and also less susceptible to restriction by rhesus TRIM5 $\alpha$ . Careful examination of standard deviation from the mean in Figure 2-2 may show that the increase we see in infectivity in Cf2-LPCX cells is in fact negligible; however, the difference in TRIM5 $\alpha$  susceptibility seems significant. Purified EIAV cores have been used previously to investigate the binding of TRIM5-21R to EIAV CA (19). Future studies should employ a similar binding assay to investigate rhesus TRIM5 $\alpha$  binding to the cores of CA mutants L87M, N89A and N89E. Reduced binding may explain the reduced sensitivity to TRIM5 $\alpha$ . The possibility also exists that the assays we used to look at uncoating and production of reverse transcripts, 2-LTR circles and integrated provirus are not sensitive enough to detect small differences that may be the basis of the increased infectivity. Finally, although we have measured persistent viral DNA two weeks post-infection, our assay does not actually measure integrated provirus, nor

does it give any information about integration sites. HIV-1 and EIAV preferentially integrate into active transcription units. Although it seems highly unlikely, one possible explanation for enhanced GFP expression in mutants L87M, N89A and N89E could be that the introduction of these amino acid changes has in some way caused them to integrate into locations where they are more highly expressed than wt EIAV. This can be examined using ligation-mediated PCR (LM-PCR), which has previously been used to determine the integration profile of wt EIAV (14).

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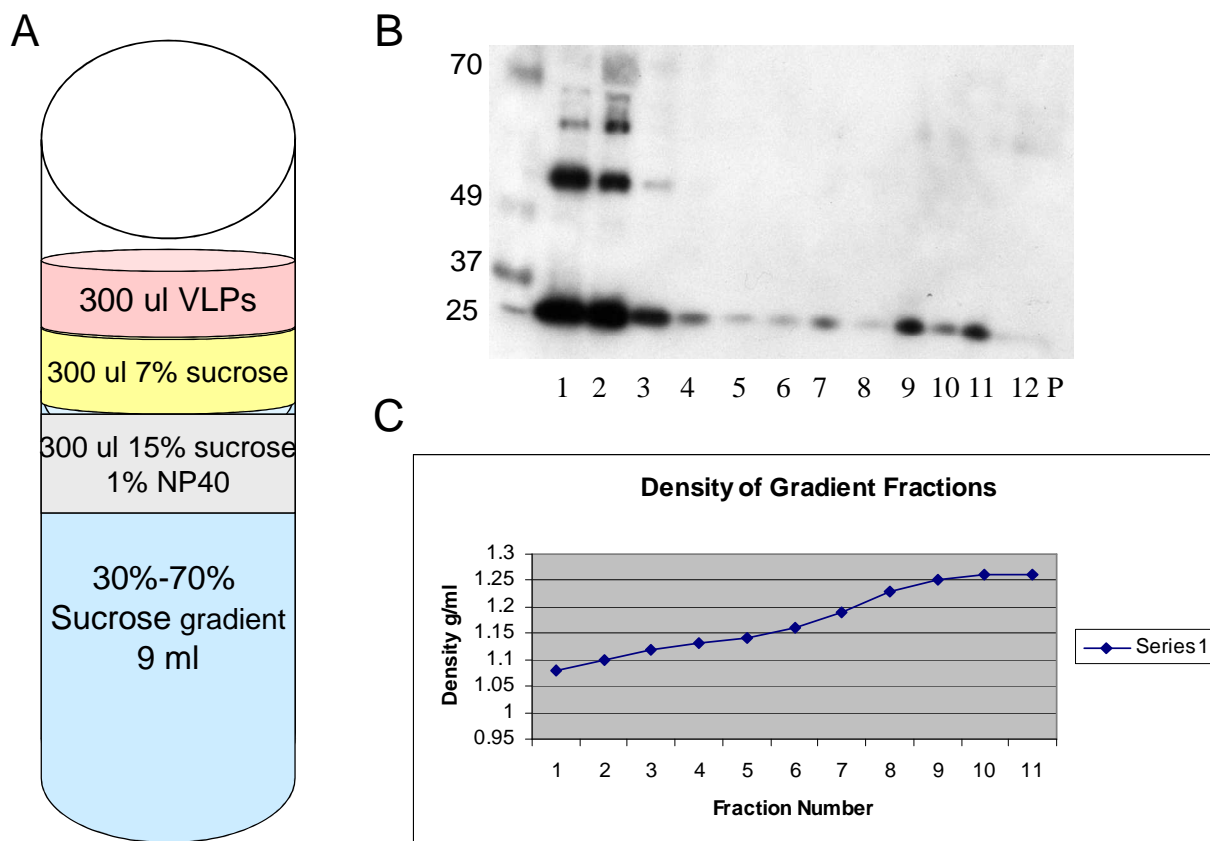
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# **APPENDIX**

Both HIV-1 and EIAV have conical capsids composed of CA protein hexamers which are arranged in the mature virion in a hexagonal lattice termed a fullerene cone (2, 4-6, 8, 11). Although intact HIV-1 core complexes can be isolated from membrane-stripped viruses and purified using sucrose-gradient centrifugation, these isolated cores are typically unstable and tend to disassemble spontaneously in buffer (1, 3, 9, 12, 13). Structural studies of HIV-1 CA have therefore typically used synthetic assemblies created from a CA-NC protein fusion (5). However, attempts to isolate EIAV core complexes virions have proven more successful. Roberts and Oroszlan were able to isolate intact cores from detergent-treated virions, and these cores maintained their characteristic cone shape under electron microscopy (12). More recently, Langelier *et al.* used purified EIAV cores to test binding of a TRIM5-21R protein to authentic purified core particles (10).

**Purification of EIAV cores from VLPs.** 293T cells were transfected with the pEV53 EIAV vector and supernatant was collected 36 hours post-transfection. Virus-like particles (VLPs) were concentrated in the SW28 rotor, 2 h at 27000 rpm. VLPs representing one T-175 flask were resuspended in 300  $\mu$ l and layered over a 300  $\mu$ l 7% sucrose cushion, a 300  $\mu$ l 15% sucrose/1% NP40 cushion, and then a 9 ml 30-70% sucrose gradient or a 9 ml 60% sucrose cushion. Samples were centrifuged in the SW41 rotor at 40000 rpm for either 16 h (gradient) or 3 h (cushion). The 60% sucrose cushion was aspirated and the pellet of purified cores was resuspended in 100  $\mu$ l of PBS. Cell lysates were prepared using a hypotonic lysis buffer and 3X freeze-thaw cycles. Cell lysates were diluted to a final concentration of 2  $\mu$ g/ml. For each sample, 250  $\mu$ l of cell lysate was added to 12  $\mu$ l of cores (the amount of purified cores added to each sample represents 1/6 of the VLPs harvested from one transfected T-175 flask, e.g. six samples = one flask). Incubations proceeded for a given amount of time at 37°C. Samples were centrifuged in an SW55 rotor, 210000xg, for 2 h. The pellet was resuspended in 100  $\mu$ l SDS buffer and 50  $\mu$ l were blotted for p26 (each gel lane represents 1/2 total pellet). Western blotting for p26 CA was done using an anti-EIAV polyclonal serum and a HRP-conjugated anti-horse IgG secondary antibody.

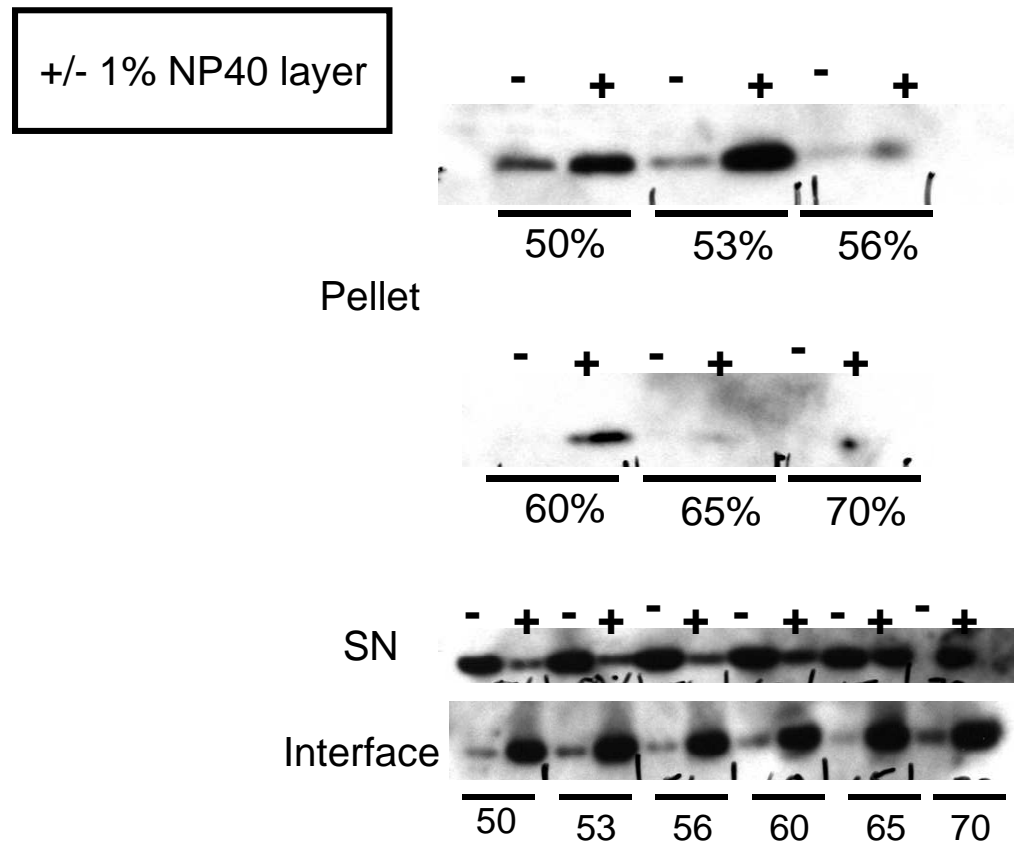
We have attempted to isolate intact EIAV cores from detergent-treated virions to use in the study of the effects of various cell lysates on CA stability. The protocol used contains slight modifications from that used by Langelier *et al.* (10). An example of successful core purification is shown in Figure A-1. Intact EIAV VLPs were created by transfecting one T-175 flask with 25 µg of the EIAV *gag-pol* vector pEV53. The VLPs were harvested 36 h post-transfection, clarified and concentrated via centrifugation, then resuspended in 300 µl of PBS. The VLPs were layered onto a 300 µl 7% sucrose cushion, which was layered on top of a 300 µl 15% sucrose cushion containing 1% NP40. Underneath the VLPs and sucrose cushions was a 9 ml 30-70% sucrose gradient. These cushion-gradients were then centrifuged in a SW41 rotor at 40,000 rpm for 16 hours. Following centrifugation, twelve fractions were collected top-down from the gradient, and the “pellet” fraction at the bottom of the tube was also resuspended. Each fraction was blotted for CA (p26) (Fig. A-1B) and the refractive index of each fraction was measured (Fig. A-1C). Fractions 8-11 had the correct density, corresponding to the density of EIAV cores (1.22-1.26 g/ml).



**Figure A-1. Purification of intact EIAV cores from detergent-treated VLPs using a sucrose gradient.** (A) Arrangement of the VLPs, detergent cushion and sucrose gradient prior to centrifugation. (B) Top-down fractions collected post-centrifugation blotted for p26 using anti-EIAV serum and an HRP-conjugated anti-horse IgG secondary antibody. (C) Fraction density measurements collected via refractometer. Fractions 8-11 have a density corresponding to that of EIAV cores.

Long sequential periods of centrifugation are required to isolate cores from a sucrose gradient. This minimizes the yield of intact complexes recovered, most likely because cores disassemble under repeated handling, resuspension and centrifugation. To minimize the centrifugation and handling time in the hopes of increasing the yield of intact cores, we attempted to purify cores using a fixed-percentage sucrose cushion rather than a gradient, with or without an overlay of detergent. Figure A-2 shows the results of the various percentages of sucrose tested (when a detergent overlay was included, the percentage of NP40 remained fixed at 1% in 15% sucrose in a volume of 300  $\mu$ l). We hoped to determine the optimal percentage of sucrose that would

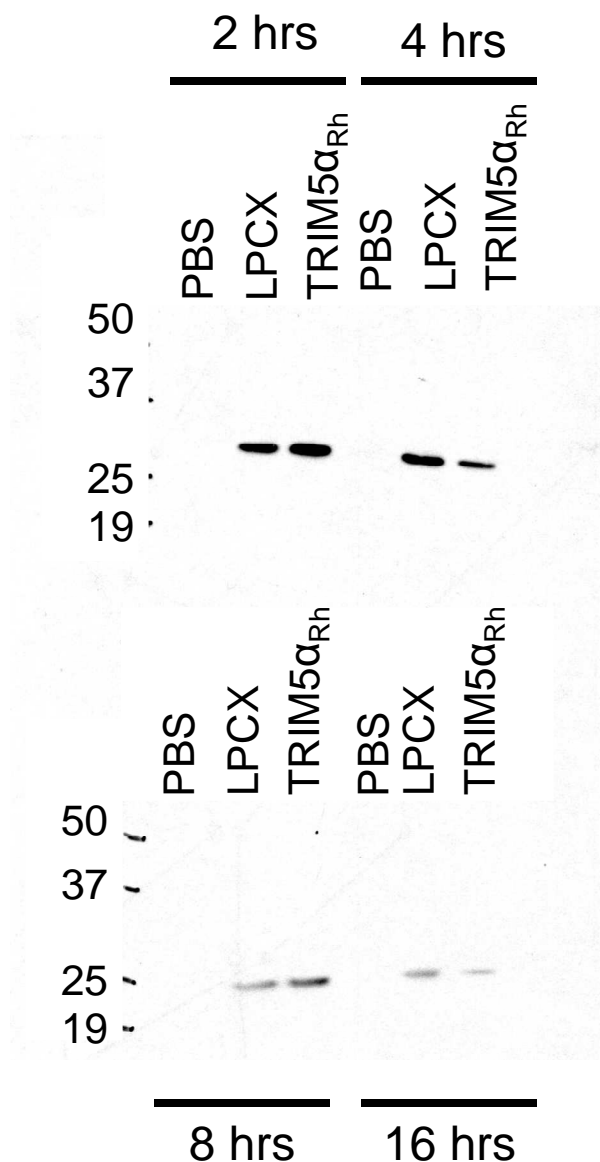
exclude enveloped VLPs from the pellet while still allowing a substantial percentage of the membrane-stripped cores to pellet. We prioritized exclusion of membrane-intact VLPs over yield and therefore chose the more stringent 60% sucrose over higher-yield 53% sucrose. All cores used for subsequent experiments were purified over a 60% sucrose cushion.



**Figure A-2. Separation of enveloped VLPs from non-enveloped cores using a fixed-percentage sucrose cushion.** A 60% sucrose cushion allows optimal separation of purified core complexes from enveloped VLPs while retaining a good yield of p26 in the pellet. SN: a fraction of supernatant taken from the extreme top of the tube post-centrifugation. Interface: a fraction taken at the meniscus of the detergent layer/sucrose cushion.

Previous work has shown that HIV-1 CA mutants that undergo rapid uncoating exhibit infectivity defects and cellular cofactors that promote rapid core disassembly (e.g. TRIM5 $\alpha$ ) also have negative effects on infectivity. Therefore, it is possible that the uncoating process may be a

passive disassembly of an inherently unstable virion core. If this is the case, then during productive infection cellular cofactors may act temporally to regulate uncoating by promoting core stability rather than disassembly. Our lab is currently investigating the possible role of PDZD8 as one such cellular stabilizing factor using HIV-1 CA-NC complexes [Alex Guth, unpublished data; (7)]. Thus far, we have seen that incubation of HIV-1 CA-NC complexes in cell lysates stabilizes them to a greater extent than incubation in buffer or PBS. We wished to investigate whether these stabilizing effects were applicable to native core complexes. Two types of cell lysates were tested: lysates from Cf2 cells stably expressing either the empty retroviral vector LPCX or stably expressing rhesus TRIM5 $\alpha$ . We hypothesized that the presence of rhesus TRIM5 $\alpha$ , which restricts EIAV infection and promotes accelerated uncoating of HIV-1 *in vivo*, might also affect core stability *in vitro*. The effects of incubation of purified cores in either of these two cell lysates are summarized in Figure A-3. Cores were incubated with either cell lysate or PBS for as long as 16 hours and were harvested after various lengths of time to develop a time course of stability. After 2 hours of incubation in PBS, the cores ceased to pellet during the post-incubation centrifugation, suggesting that they had disassembled. However, cores incubated with either type of cell lysate continued to pellet up to 16 hours after they were added to the lysate. The amount of particulate CA representing intact cores began to decrease at some point between 4-8 hours, as measured by Western blotting for p26 CA in the pellet. The presence of rhesus TRIM5 $\alpha$  in the cell lysate did not appear to affect stability, suggesting that the presence of rhesus TRIM5 $\alpha$  *in vitro* does not abrogate the stabilizing effect of the cell lysate.

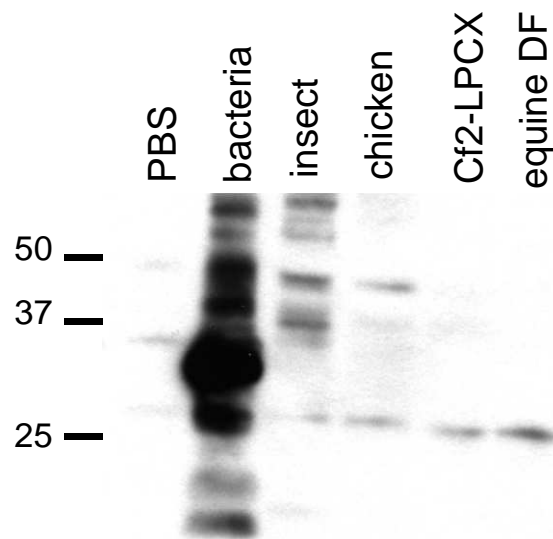


**Figure A-3. A time course of core stability in PBS, cell lysates, or cell lysates containing TRIM5 $\alpha_{Rh}$ .** Purified cores were incubated for a given amount of time with either PBS or cell lysates at 37°C. Post-incubation, particulate CA protein was pelleted by centrifugation and the pellet resuspended. Each pellet was Western blotted for p26.

We also wished to test whether the stabilizing effect we observed with the Cf2 cell lysates was conserved across different eukaryotic cell types or possibly even prokaryotic cells. Purified cores were incubated with cell lysates prepared from DH5 $\alpha$  bacteria, SF9 insect cell lysate,



chicken cell lysate, Cf2-LPCX cell lysate, and lysate from equine dermal (ED) cells. As a negative control, cores were also incubated with PBS. The incubation of cores with lysates proceeded for 4 hours. Figure A-4 shows the stabilizing effects of the various lysates. It is difficult to interpret whether the bacterial lysate stabilized the cores. To avoid the introduction of any detergent into the reaction, which could lead to core disassembly, bacteria were lysed by sonication alone and the lysate clarified by centrifugation. However, several proteins which were nonspecifically recognized by the anti-EIAV serum remained in the lysate. It appears that all the remaining eukaryotic cell lysates do stabilize the cores to some extent, and all of them stabilize more than PBS.



**Figure A-4. Lysates from a variety of eukaryotic cells stabilize purified EIAV cores.** Purified cores were incubated for 4 h at 37°C in PBS or various cell lysates. Following incubation, the reaction was centrifuged to pellet intact cores. The pellet was resuspended and blotted for p26 CA. Bacteria: DH5 $\alpha$ ; insect: SF9; Cf2-LPCX: canine thymocytes; equine DF: dermal fibroblasts.

## Conclusions

The results obtained with the purified EIAV CA cores corroborate results our lab has obtained from experiments investigating the stability of HIV-1 CA-NC complexes. For both EIAV cores and HIV-1 CA-NC assemblies, we see that cell lysates stabilize the CA complexes better than PBS, the presence of the restriction factor TRIM5 $\alpha_{Rh}$  does not affect complex stability, and the stabilizing effect is seen with a variety of eukaryotic cell lysates. Current work in our lab is focused on the possible identity of PDZD8 as the protein responsible for this stabilizing effect. We are using both purified EIAV cores and HIV-1 CA-NC assemblies in these experiments. The experiments above have shown that EIAV cores can easily be purified and are reliable markers of capsid stability for *in vitro* experiments. This method of core purification will be useful in any study examining the stability of authentic, purified core particles *in vitro* and also can be used to support findings involving the stability of HIV-1 CA-NC complexes. In addition, purified EIAV cores can be used in assays investigating the binding of other proteins to intact EIAV CA in its native form, as has previously been described (10).

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